IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)
	)
Philippe SANSONETTI et al.	) Group Art Unit: 1645
	)
Application No.: 08/466,698	) Examiner: Albert M. NAVARRO
	)
Filed: June 6, 1995	)
	)
For: METHOD FOR PRODUCING	)
TRANSFORMED *SHIGELLA* (As	)
Amended)	)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION OF STEWART THOMAS COLE, PH.D., UNDER 37 C.F.R. § 1.132

I, Stewart Thomas Cole, Ph.D., do hereby make the following declaration:

1.   My curriculum vitae, including a list of publications that I have authored or coauthored, is attached hereto as Exhibit A.

2.   I have been employed at Institut Pasteur, in Paris, France, since 1983. Since 1989, I have been Chief of the Bacterial Molecular Genetics unit. During the period of 1983-1988, my work was focused on bacterial molecular genetics.

3.   On information and belief, attached hereto as Exhibit B is a copy of U.S. patent application Serial No. 08/466,698 ("the '698 application").

4.   I have read and understood the contents of the attached copy of the '698 application.

5.   On information and belief, the '698 application was filed on June 6, 1995.

6.   On information and belief, the '698 application claims the benefit of priority of European Patent Application Serial No. 88 401 842.5, which was filed on July 15, 1988.

7.   On information and belief, attached hereto as Exhibit C is a copy of "Proposed Claims for U.S. Patent Application Serial No. 08/466,698" ("the proposed claims").

8.   On information and belief, attached hereto as Exhibit D is a copy of a journal article published in the August 15, 1986, issue of Cell, authored by Makino et al., and entitled "A Genetic Determinant Required for Continuous Reinfection of Adjacent Cells on Large Plasmid in *S. flexneri* 2a" ("Makino").

9.   I have read and understood the contents of the attached copy of Makino.

10.   I am submitting this Declaration to explain the significance of certain terms that appear in the proposed claims to describe modified *Shigella*. In particular, I am submitting this Declaration to explain how these terms in the proposed claims would have been understood on July 15, 1988. In considering the meaning of the terms, I have considered the disclosure of the '698 application.

11.   As described in the '698 application, Shigellosis (also known as bacillary dysentery) is a disease endemic throughout the world. (Exhibit B at page

2

1, lines 14-22.) The primary step in the pathogenesis of bacillary dysentery is invasion of the human colonic mucosa by *Shigella* bacteria. (Exhibit B at page 1, lines 23-24.) Mucosal invasion encompasses many steps, including penetration of the bacteria into epithelial cells, intracellular multiplication, killing of host cells, and finally spreading to adjacent cells and to connective tissue. (Exhibit B at page 1, lines 25-29.) The result is a strong inflammatory reaction that leads to abscesses and ulcerations of the mucosal surface. (Exhibit B at page 1, lines 29-32.) Shigellosis may lead to toxic megacolon, leukemoid reactions and hemolytic-uremic syndrome, which can be fatal. (Exhibit B at page 2, lines 5-8.)

12. The '698 application describes modified strains of *Shigella* that can be used to make a vaccine against a wild strain of *Shigella*. (Exhibit B at page 1, lines 4-13 and page 5, lines 8-34.) Although the '698 application does not use the term, this type of strain was known as of July 15, 1988, as it is today, as a "live attenuated strain". For a live attenuated strain to function effectively as a vaccine, the strain must be modified by mutation of one or more genes to eliminate its pathogenicity, but not the ability of the strain to elicit a protective immune response. Such strains were known as of July 15, 1988. For example, the "International Dictionary of Medicine and Biology", published in 1986, defined an "attenuated vaccine" at page 3083 as "A live bacterial or viral vaccine, carrying mutations that eliminate its pathogenicity but not its ability to elicit a protective immune response." (A copy of the relevant pages of International Dictionary of Medicine and Biology (1986) is attached hereto as Exhibit E.)

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13.   As of July 15, 1988, it was known that making a live attenuated *Shigella* strain would require modifying a wild *Shigella* strain by mutating one or more genes required for pathogenicity of the wild strain, to create a modified strain that will invade and multiply in a host, but, unlike the corresponding wild strain, will not cause a disease pathology. It was appreciated that, while attenuation of the live attenuated strain is critical to render the strain non pathogenic, it is imperative that the strain retain some ability to invade, multiply, and spread within an inoculated host, so that the strain elicits a significant enough immune response to confer immunity to the wild strain to the host.

14.   Proposed claim 24 recites "[a] method for modifying a wild strain of an enteroinvasive *Shigella* to produce a modified strain of *Shigella* that can not spread substantially within infected cells of a host and can not spread substantially from infected to uninfected cells of the host, for use in making a vaccine against the wild strain of *Shigella*. . . ." I interpret this language to mean that the ability of the strain to spread within infected host cells, and from infected to uninfected host cells, is substantially reduced. However, the ability of the strain to spread within infected host cells, and from infected to uninfected host cells, is clearly not abolished. If it were, the modified strain would not be useful to make a vaccine against the wild strain of *Shigella*.

15.   Similarly to proposed claim 24, each of proposed claims 36, 37, 54, 55, 74, and 79 recites a modified *Shigella* that "can not spread substantially within infected cells of a host and can not spread substantially from infected to uninfected cells of the host." Each of these claims recites "[a] modified *Shigella* for use in

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making a vaccine against a wild strain of *Shigella*." I interpret this language in each of these claims to mean that the ability of the modified strain to spread within infected host cells, and from infected to uninfected host cells, is substantially reduced. However, the ability of the strain to spread within infected host cells, and from infected to uninfected host cells, is clearly not abolished. If it were, the modified strain would not be useful to make a vaccine against the wild strain of *Shigella*.

16.   Proposed claim 25, as it depends from proposed claim 24, recites that the modified strain of *Shigella* produced by the method "can not spread substantially within infected cells of a host and can not spread substantially from infected to uninfected cells of the host" and "also can not substantially invade cells of the host." I interpret this language to mean that the ability of the modified strain to spread within infected host cells, the ability of the strain to spread from infected to uninfected host cells, and the ability of the strain to invade host cells are all substantially reduced. However, the ability of the modified strain to spread within infected host cells, the ability of the strain to spread from infected to uninfected host cells, and the ability of the strain to invade host cells, are clearly not abolished. If they were, the modified strain would not be useful to make a vaccine against the wild strain of *Shigella*.

17.   Similarly to proposed claim 25, each of proposed claims 36, 37, 54, and 55, recites a modified *Shigella* that "can not spread substantially within infected cells of a host and can not spread substantially from infected to uninfected cells of the host" and "also can not substantially invade cells of the host." Each of

these claims recites "[a] modified *Shigella* for use in making a vaccine against a wild strain of *Shigella*." I interpret this language to mean that the ability of the modified strain to spread within infected host cells, the ability of the strain to spread from infected to uninfected host cells, and the ability of the strain to invade host cells are all substantially reduced. However, the ability of the modified strain to spread within infected host cells, the ability of the strain to spread from infected to uninfected host cells, and the ability of the strain to invade host cells, are clearly not abolished. If they were, the modified strain would not be useful to make a vaccine against the wild strain of *Shigella*.

18.   Proposed claim 26, as it depends from proposed claim 25, recites that the modified strain of *Shigella* produced by the method "can not spread substantially within infected cells of a host and can not spread substantially from infected to uninfected cells of the host" and "also can not substantially invade cells of the host" and "also can not produce toxins that kill a substantial number of the host's cells." I interpret this language to mean that the ability of the modified strain to spread within infected host cells, the ability of the strain to spread from infected to uninfected host cells, the ability of the strain to invade host cells, and the ability of the strain to produce toxins that kill a substantial number of the host's cells are all substantially reduced. However, the ability of the modified strain to spread within infected host cells, the ability of the strain to spread from infected to uninfected host cells, the ability of the strain to invade host cells, and the ability of the strain to produce toxins that kill a substantial number of the host's cells, are

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clearly not abolished. If they were, the modified strain would not be useful to make a vaccine against the wild strain of Shigella.

19.   Similarly to proposed claim 26, each of proposed claims 37 and 55 recites a modified Shigella that "can not spread substantially within infected cells of a host and can not spread substantially from infected to uninfected cells of the host" and "also can not substantially invade cells of the host" and "also can not produce toxins that kill a substantial number of the host's cells." Each of these claims recites "[a] modified Shigella for use in making a vaccine against a wild strain of Shigella." I interpret this language to mean that the ability of the modified strain to spread within infected host cells, the ability of the strain to spread from infected to uninfected host cells, the ability of the strain to invade host cells, and the ability of the strain to produce toxins that kill a substantial number of the host's cells are all substantially reduced. However, the ability of the modified strain to spread within infected host cells, the ability of the strain to spread from infected to uninfected host cells, the ability of the strain to invade host cells, and the ability of the strain to produce toxins that kill a substantial number of the host's cells, are clearly not abolished. If they were, the modified strain would not be useful to make a vaccine against the wild strain of Shigella.

20.   I have read the Declaration of Jean-Michel Alonso, M.D., Ph.D., Under 37 C.F.R. § 1.132, a copy of which is attached hereto as Exhibit F. I agree with Dr. Alonso that, based on the teachings of Makino, I would not expect that a modified Shigella strain comprising an inactivated icsA gene would be useful as a live attenuated strain for making a vaccine against the wild Shigella strain.

21. In reaching this conclusion, I am aware that Makino states that modified *Shigella* strains comprising an inactivated *icsA* gene "may be a plausible candidate for a live vaccine against bacillary dysentery." (Exhibit D at page 554, left col.) I disagree with this statement. In fact, this assertion is clearly contrary to the description of the modified *Shigella* strain comprising an inactivated *icsA* gene provided by Makino. According to Makino, the modified strain is unable to survive in cells or tissues and does not spread within or between cells. (Exhibit D at page 551, right col. and page 554, left col.) For this reason, the strain would not elicit a robust immune response and would not be effective for making a vaccine.

22. Based on the disclosure in Makino, and based on what was known as of July 15, 1988, and absent reading the disclosure of the '698 application, I would not have been motivated to include an inactivated *icsA* gene in a modified *Shigella* strain for use in making a vaccine. To the contrary, I would have assumed that inclusion of an inactivated *icsA* gene in such a strain would have rendered it ineffective in making a vaccine against a wild strain of *Shigella*.

23. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the

application or any patent issuing thereon.

Dated: _____   By:_____
                                 Stewart Thomas Cole, Ph.D.

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21.   In reaching this conclusion, I am aware that Makino states that modified *Shigella* strains comprising an inactivated *icsA* gene "may be a plausible candidate for a live vaccine against bacillary dysentery." (Exhibit D at page 554, left col.) I disagree with this statement. In fact, this assertion is clearly contrary to the description of the modified *Shigella* strain comprising an inactivated *icsA* gene provided by Makino. According to Makino, the modified strain is unable to survive in cells or tissues and does not spread within or between cells. (Exhibit D at page 551, right col. and page 554, left col.) For this reason, the strain would not elicit a robust immune response and would not be effective for making a vaccine.

22.   Based on the disclosure in Makino, and based on what was known as of July 15, 1988, and absent reading the disclosure of the '698 application, I would not have been motivated to include an inactivated *icsA* gene in a modified *Shigella* strain for use in making a vaccine. To the contrary, I would have assumed that inclusion of an inactivated *icsA* gene in such a strain would have rendered it ineffective in making a vaccine against a wild strain of *Shigella*.

23.   I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the

application or any patent issuing thereon.

Dated: 16.1. 204

By: _____

Stewart Thomas Cole, Ph.D.

CURRICULUM VITAE

Stewart Thomas COLE

Professor at the Pasteur Institute

Chef de l'Unité de Génétique Moléculaire Bactérienne
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Date of birth: 14 January 1955 (Penzance, U.K.)

Nationality: British

Higher Education

1976	Bachelor of Science, University of Wales (Microbiology)
1979	Doctor of Philosophy, University of Sheffield (Molecular Genetics)

Research Career

1979-1980 Sweden	Post-doctoral fellow at the University of Umeå, (Prof. S. NORMARK),
1980-1983	Wissenschaftlicher Assistent, (Prof. U. HENNING), Max-Planck Institute für Biologie, Tübingen, Germany
1983-1986	Chargé de Recherche à l'Institut Pasteur, Groupement de Génie Génétique, (Dr. R.E. STREECK)
1986-1988	Chargé de Recherche à l'Institut Pasteur, Biochimie des Régulations Cellulaires (Prof. A. ULLMANN)
1989-	Chef de l'Unité de Génétique Moléculaire Bactérienne, à l'Institut Pasteur
1998-	Professeur à l'Institut Pasteur
2000-	Directeur des Equipements et Technologies Stratégiques, à l'Institut Pasteur

Distinctions and awards

2001	Prix Jean-Pierre Lecocq, Academie des Sciences, France
2001	Prix Thérèse Lebrasseur, Fondation de France, France
2002	Marjory Stephenson Prize Lecture, Society for General Microbiology, UK EMBO Fellow

Other Professional Activities

Advisor to the THELEP, IMMLEP, THEMYC, and IMMYC Steering Committees (1987-1993) of the World Health Organisation (WHO);

Member of the Editorial Board, *Infection and Immunity* (1994-1996), *Journal of Bacteriology* (1995-1997), *Archives in Microbiology* (1994-1999), *Tubercle and Lung Disease* (1998-);

Ad hoc reviewer for *Nature, Science, Cell, Molecular Microbiology, etc.*;

Member of the IMMYC (1997-) and New Vaccination Approaches (1998-) Steering Committees at WHO;

Advisor to the *European Union, DGXII, Deutsche Forschungsanstalt für Luft- und Raumfahrt, Schwerpunkt Infektionsforschung, National Institute of Allergy and Infectious Diseases, NIH;*

Member of *la Commission Scientifique Spécialisée* INSERM n° 10 (Infectious Diseases 1994-1998);

Ad hoc grant reviewer: Wellcome Trust, NIH, NSF, EU, BBSRC, MENRT, etc.;

Member of Scientific Advisory Boards: the *Institut Pasteur de Lille* (1996-), the *Institut Biologique de Lille* (1997-), and the *Centre National de Séquençage* (1997-2002; 2003-..); Wellcome Trust Sanger Institute, PSAG (2002-..)

Visiting Professor, Imperial College School of Medicine at St Mary's, London (1996-98).

Publications
(twenty most significant papers are shown in bold face)

1. Cole, S. T. and J. R. Guest (1979) Production of a soluble form of fumarate reductase by multiple gene duplication in *Escherichia coli* K12. European Journal of Biochemistry, **102**:65-71.

2. Cole, S. T. and J. R. Guest (1979) Amplification and aerobic synthesis of fumarate reductase in ampicillin-resistant mutants of *Escherichia coli* K12. FEMS Microbiol. Lett., **5**:65-67.

3. Cole, S. T. and J. R. Guest (1980) Genetic and physical characterization of lambda transducing phages (lambda *frdA*) containing the fumarate reductase gene of *Escherichia coli* K12. Molecular & General Genetics, **178**:409-18.

4. Cole, S. T. and J. R. Guest (1980) Amplification of fumarate reductase synthesis with lambda *frdA* transducing phages and orientation of *frdA* gene expression. Molecular & General Genetics, **179**:377-85.

5. Guest, J. R., S. T. Cole and K. Jeyaseelan (1981) Organization and expression of the pyruvate dehydrogenase complex genes of *Escherichia coli* K12. Journal of General Microbiology, **127**:65-79.

6. Cole, S. T., I. Sonntag and U. Henning (1982) Cloning and expression in *Escherichia coli* K-12 of the genes for major outer membrane protein OmpA from *Shigella dysenteriae, Enterobacter aerogenes,* and *Serratia marcescens.* Journal of Bacteriology, 149:145-50.

7. ***Cole, S. T. (1982) Nucleotide sequence coding for the flavoprotein subunit of the fumarate reductase of *Escherichia coli.* European Journal of Biochemistry, 122:479-84.**

8. Braun, G. and S. T. Cole (1982) The nucleotide sequence coding for major outer membrane protein OmpA of *Shigella dysenteriae*. Nucleic Acids Research, **10**:2367-78.

9. Cole, S. T., G. Braun, I. Sonntag and U. Henning (1982) Expression in *Escherichia coli* K12 of the cloned genes for a major outer membrane protein (OmpA protein) from *Shigella dysenteriae, Enterobacter aerogenes* and *Serratia marcescens*. Annales de Microbiologie, **133**:209-13.

10. Cole, S. T. and J. R. Guest (1982) Molecular genetic aspects of the succinate: fumarate oxidoreductases of *Escherichia coli*. Biochemical Society Transactions, **10**:473-5.

11. Bremer, E., S. T. Cole, I. Hindennach, U. Henning, E. Beck, C. Kurz and H. Schaller (1982) Export of a protein into the outer membrane of *Escherichia coli* K12. Stable incorporation of the OmpA protein requires less than 193 amino-terminal amino-acid residues. European Journal of Biochemistry, **122**:223-31.

12. Cole, S. T., E. Bremer, I. Hindennach and U. Henning (1982) Characterisation of the promoters for the *ompA* gene which encodes a major outer membrane protein of Escherichia coli. Molecular & General Genetics, **188**:472-9.

13. Cole, S. T., T. Grundstrom, B. Jaurin, J. J. Robinson and J. H. Weiner (1982) Location and nucleotide sequence of *frdB*, the gene coding for the iron-sulphur protein subunit of the fumarate reductase of *Escherichia coli*. European Journal of Biochemistry, **126**:211-6.

14. Braun, G. and S. T. Cole (1983) Molecular characterization of the gene coding for major outer membrane protein OmpA from *Enterobacter aerogenes*. European Journal of Biochemistry, **137**:495-500.

15. Cole, S. T. (1983) Characterisation of the promoter for the LexA regulated *sulA* gene of *Escherichia coli*. Molecular & General Genetics, **189**:400-4.

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Transformed Shigella

Background of the Invention

This invention relates to a method of modifying the genome of an entero-invasive wild strain of Shigella so that the strain cannot substantially invade cells and tissues of an infected host and cannot spread substantially within infected cells and between infected and non-infected cells of the host and cannot produce toxins which will kill substantial numbers of the hosts' cells. This invention particularly relates to such a modified strain of Shigella which can be used to immunize a host against the wild strain of Shigella.

Shigellosis or bacillary dysentery is a disease that is endemic throughout the world. The disease presents a particularly serious public health problem in tropical regions and developing countries where Shigella dysenteriae 1 and S. flexneri predominate. In industrialized countries, the principal etiologic agent is S. sonnei although sporadic cases of shigellosis are encountered due to S. flexneri, S. boydii and certain entero-invasive Escherichia coli.

The primary step in the pathogenesis of bacillary dysentery is invasion of the human colonic mucosa by Shigella (23). Mucosal invasion encompasses several steps which include penetration of the bacteria into epithelial cells, intracellular multiplication, killing of host cells, and final spreading to adjacent cells and to connective tissue (9, 41, 55, 56). The overall process which is usually limited to the mucosal surface leads to a strong inflammatory reaction which is responsible for abscesses and ulcerations (23, 41, 55).

Even though dysentery is characteristic of shigellosis, it may be preceded by watery diarrhea. Diarrhea appears to be the result of disturbances in

colonic reabsorption and increased jejunal secretion whereas dysentery is a purely colonic process (20, 41). Systemic manifestations may also be observed in the course of shigellosis, mainly in the cases due to S. dysenteriae 1. These include toxic megacolon, leukemoid reactions and hemolytic-uremic syndrome ("HUS"). The latter is a major cause of mortality from shigellosis in developing areas (11, 22, 38).

The role of Shiga-toxin produced at high level by S. dysenteriae 1 (6) and Shiga-like toxins ("SLT") produced at low level by S. flexneri and S. sonnei (19, 30) in the four major stages of shigellosis (i.e., invasion of individual epithelial cells, tissue invasion, diarrhea and systemic symptoms) is not well understood. For review see O'Brien and Holmes (32). Plasmids of 180-220 kilobases ("kb") are essential in all Shigella species for invasion of individual epithelial cells (41, 42, 44). This includes entry, intracellular multiplication and early killing of host cells (4, 5, 46). The role of Shiga-toxin and SLT at this stage is unclear. They do not appear to play a crucial role in intracellular multiplication and early killing (4, 12, 46). However none of the experiments which have been carried out has compared isogenic mutants in a relevant cell assay system. Recent evidence indicates that Shiga-toxin is cytotoxic for primary cultures of human colonic cells (27). Tissue invasion requires additional chromosomally encoded products among which are smooth lipopolysaccharides ("LPS") (44, 57), the non-characterized product of the Kcp locus (8, 44), and aerobactin (24, 28). A region of the S. flexneri chromosome necessary for fluid production in rabbit ileal loops has been localized to the rha-mtl regions and near the lysine decarboxylase locus (44). However, no evidence has been adduced to show that the ability to cause fluid

accumulation is due to the SLT of S. flexneri. Thus, the role of Shiga-toxin in causing the systemic complications of shigellosis is still hypothetical. However, Shiga-toxin can mediate vascular damage since capillary lesions observed in HUS resemble those observed in cerebral vessels of animals injected with this toxin (1, 2, 22).

A mutant which lacks Shiga-toxin or SLT could indicate the role of these toxins in the disease process. S. dysenteriae 1, which produces the highest amount of this cytotoxin, could be transformed into such a Shiga-toxin negative mutant ("Tox-") and could serve best to indicate the role of the toxin -- despite Sekizaki et al's (48) having obtained such a mutant which appeared as invasive in the HeLa cell assay and the Sereny test (49) as the wild strain. More importantly, such a Tox⁻ mutant could be used to make a mutant which could not invade, and then multiply substantially within, cells of a host and also could not spread substantially within the host's infected cells and from there to the host's uninfected cells and also could not produce toxins which would kill subtantial numbers of infected, as well as uninfected, host cells. As a result, the Tox⁻ mutant could be used to immunize a host against a wild strain of the Shigella.

SUMMARY OF THE INVENTION

A Tox⁻ mutant of a wild strain of S. dysenteriae 1 is genetically engineered by allelic exchange with an in vitro mutagenized Shiga-toxin gene. The effect of this mutation in cell assay systems and animals shows that the mutant can be genetically engineered further to provide a mutant which cannot substantially invade and then spread within and between host cells and cannot produce Shiga-toxins in host cells.

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Also in accordance with the invention, the Tox⁻ mutant of the wild strain of S. dysenteriae 1 is genetically engineered further by allelic exchange with:

a) an in vitro mutagenized gene of S. dysenteriae 1 which encodes a protein necessary for S. dysenteriae 1 to invade a host's cells, as well as tissues, such as a gene which codes for a protein necessary for the chelation of iron and/or the transport of iron into S. dysenteriae 1 (e.g., an enterobactin or enterochelin gene of S. dysenteriae 1); and

b) an in vitro mutagenized gene of S. dysenteriae 1 which encodes a protein necessary for S. dysenteriae 1 to spread within infected cells and between infected and uninfected cells, such as an intra-intercellular spread gene (e.g., an ics A or vir G gene).

Further in accordance with this invention, a mutant of a wild strain of S. flexneri is genetically engineered by allelic exchange with: a) an in vitro mutagenized gene of S. flexneri which encodes a protein necessary for S. flexneri to invade a host's cells, as well as tissues, such as a gene which codes for a protein necessary for the chelation of iron and/or the transport of iron into S. flexneri (e.g., an aerobactin gene of S. flexneri); and b) an in vitro mutagenized gene which encodes a protein necessary for S. flexneri to spread within and between the host's cells, such as an ics A gene.

Still further in accordance with this invention, the mutants of Shigella of this invention are used for making vaccines against the wild strains of Shigella.

BRIEF DESCRIPTION OF THE FIGURE

The Figure shows schematically the cloning of the Shiga-toxin operon and in vitro mutagenesis of the

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<u>Shiga</u>-toxin A subunit gene in Example 2. In plasmids pHS7201, pHS7202 and pHS7203 in the Figure: Solid lines indicate sequences from the A subunit gene; Stippled lines indicate B subunit gene sequences; and Stripped lines indicate sequences from the Ω insertion element.

DETAILED DESCRIPTION OF THE INVENTION

A method is provided for modifying a wild strain of an entero-invasive <u>Shigella</u> so that the modified strain can be used for making a vaccine against the wild strain of <u>Shigella</u>. The wild strain of <u>Shigella</u> is modified so that it cannot invade and then multiply substantially within infected cells of a host, particularly a human host, and cannot spread substantially within infected cells and from infected to uninfected cells of the host and cannot produce toxins which will kill substantial numbers of the host's infected, as well as uninfected, cells. The method involves transforming the genome, (e.g., the large virulence plasmid pHS7200) of the wild strain of <u>Shigella</u>, such as an <u>S</u>. <u>flexneri</u>, so that gene(s) of the wild strain, coding for one or more proteins necessary for the strain to invade an infected host's cells, as well as tissues (e.g., an aerobactin gene), and coding for one or more proteins necessary for the strain to spread within and between the infected host's cells (e.g., an <u>ics</u> A gene [60, 61]), are wholly or partly removed or permanently inactivated, preferably at least partly removed. For transforming the genome of a wild strain such as a <u>S</u>. <u>dysenteriae</u> 1, the method preferably involves also wholly or partly removing or permanently inactivating, preferably at least partly removing, the gene(s), preferably just the A subunit gene, coding for <u>Shiga</u>-toxin.

In the method of this invention, the genes of the wild strain of <u>Shigella</u> can be wholly or partly removed

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or permanently inactivated in a conventional manner, for example by allelic exchange with _in vitro_ mutagenized genes, at least significant portions of which preferably have been removed. In this regard, it is preferred that the mutagenized genes not be simply inactivated by means of transposons which are inserted into the genes and which can be lost by the genes when they are reproduced _in vivo_ in subsequent _Shigella_ generations when making vaccines of this invention. Rather, the mutagenized genes preferably have had significant portions thereof deleted, and suitable vaccine-compatible marker genes are preferably inserted within such deletions. Such marker genes permit so-transformed _Shigella_ to be easily identified. The preferred marker genes are the heavy metal-resistance genes such as the mercury, arsenate, arsenite, antimony, cadmium, zinc and/or cobalt-resistance genes (62, 63, 64, 65).

The cells of the modified strain can be cultured and then attenuated in a conventional manner. The cells can then be mixed with conventional pharmaceutically acceptable vehicles (e.g., an aqueous saline solution) and optionally with conventional excipients (e.g., a pharmaceutically acceptable detergent) to form a vaccine against the wild strain. The vaccine can be formulated to contain a final concentration of cell material in the range of 0.2 to 5 mg/ml, preferably 0.5 to 2 mg/ml. After formulation, the vaccine can be incorporated into a sterile container which is then sealed and stored at a low temperature (e.g., 4°C), or it can be freeze dried.

In order to induce immunity in a human host to a wild strain of _Shigella_, one or more doses of the vaccine, suitably formulated, can be administered in doses containing about 10^9-10^{11} lyophilized _Shigella_ cells. The vaccine can be administered orally in a conventional manner. The treatment can consist of a

single dose of vaccine or a plurality of doses over a period of time.

The Examples, which follow, illustrate this invention.

EXAMPLES

Unless otherwise indicated, the cloning and transformation procedures and techniques used in the Examples are the same as are generally described in Maniatis et al, "Molecular Cloning -- A Laboratory Manual", Cold Spring Harbor Laboratory (1982).

The strains, used in Example 1-6, and their phage or plasmid content are set forth in Table I.

Two media were used in the Examples: M9 minimal medium ($Na_2HPO_4.12H_2O$: 15 g/l, KH_2PO_4: 3 g/l, NaCl: 0.5 g/l, NH_4Cl: 1 g/l, $MgSO_4.7H_2O$: 0.05 g/l) and Trypto Casein Soja Broth (Diagnostics Pasteur, Marnes la Coquette, France).

Example 1 -- Cloning of the Shiga-toxin operon

Total DNA was prepared (50) from a wild type antibiotic-sensitive S. dysenteriae 1 strain SC500 obtained from Centre National de Référence des Shigelles of Institut Pasteur, Paris, France. 10 μg of DNA were digested with EcoRI (Amersham, Buckinghamshire, UK) and loaded on a 0.7% agarose gel. Fragments ranging from 3.5 to 4.5 kb were electroeluted. 0.1 μg of purified fragments was ligated to 1 μg of cos-ligated, EcoRI cut, dephosphorylated λ GT11 arms (Stratagene Cloning System, San Diego, USA) and packaged using Packagene System (Progema Biotec, Madison, USA) according to the suppliers recommendations. The packaged DNA was then transfected into E. coli Y1090(59). The λ GT11 bank was then screened with 13C4, a monoclonal antibody specific for the B subunit of SLT1 (54) obtained from A.D. O'Brien,

U.S.U.S.H., Bethesda, MD, USA. 10^3 recombinant phages were plated on Y1090 in LB soft agar. Plates were incubated at 37°C for 12 hours. A nitrocellulose filter (Schleicher and Schüll, Dassel, FRG), previously dipped into a 10 mM isopropylthiogalactoside ("IPTG") solution (Sigma, St Louis, MO, USA) was applied to the plate which was then incubated at 42°C for 2.5 hours. The filter was removed from the plate and incubated 1 hour at 37°C in PBS-milk (50 g/l dehydrated low-fat milk in 1 x PBS), washed five times with 1 x PBS, and incubated for 1 hour with the 13C4 monoclonal antibody in its non-diluted hybridoma cell supernatant. After five washes in PBS-milk, the filter was incubated 1 hour at 37°C in PBS-milk containing a 1/200 dilution of sheep anti-mouse IgG antibody conjugated with alkaline phosphatase (Biosys, Compiègne, France). The filter was washed again in 1 x PBS and placed in the staining solution: 0.33 mg/l nitro-blue tetrazolium, 0.16 mg/l 5-bromo-4-chloro-3-indolyl phosphate (both compounds from Sigma), 100 mM Tris HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$. Positive clones were plaque purified and transfected into Y1089 (59). DNA was then prepared from the lysogen (13). Subcloning was done in the EcoRI site of plasmid vector pUC8 in E. coli JM83 (58). Subclones of E. coli JM83 were tested with monoclonal antibody 13C4 as described above with the following modifications: a dry nitrocellulose filter was applied onto the plate and 2ml of a 2mg/l polymyxin B solution in PBS were added on top of the filter. The plate was then incubated at 37°C for 45 minutes before starting PBS-milk incubation. Subclone pHS7201 in E. coli JM83, containing the B subunit of SLT1, was identified.

Subclone pHS7201 of E. coli JM83 was found to have a stronger signal in colony immunoblot assay in the presence of 13C4 monoclonal antibody than parental strain

SC500 due to the gene dosage effect. A restriction map of the _Shiga_-toxin coding region within pHS7201 was identical to that of SLT1 (14). The A subunit gene was seen to possess a unique _Hpa1_ site located 310 bp

5   downstream from the ATG starting codon where a cassette could be inserted as described in Example 2.

Example 2 -- In vitro mutagenesis of the Shiga-toxin A subunit gene

10   In subclone pHS7201, the entire _Shiga_-toxin operon is contained in a 4.2kb _EcoRI_ DNA fragment. _In vitro_ mutagenesis of the A subunit gene was done by inserting the interposon Ω(37) which codes for spectinomycin resistance and is flanked on each side by T4 translation

15 transcription stop-signals. Ω was purified as an _HindIII_ 2kb fragment, and its ends were filled in by the Klenow fragment of DNA polymerase I. Ω was then ligated to _HpaI_ linearized pHS7201 to generate the recombinant plasmid pHS7202 as shown in the Figure. The 6.2kb _EcoRI_ fragment

20 containing the mutagenized sequence was then purified and ligated with the _EcoRI_ site of the suicide plasmid vector pJM703.1 (51) to generate recombinant plasmid pHS7203 as shown in the Figure. pJM703.1 replicates only if its deficient R6K origin is complemented in-trans by the pir

25 function contained in the lambda phage integrated in the genome of _E. coli_ SM10 (21). This strain also contains the transfer genes of the broad host range IncP-type plasmid RP4 integrated in its chromosome. pJM703.1 can thus be mobilized by SM10 λ pir (21) because it contains

30 the Mob site from RP4 (51). pHS7203 was thus stably maintained in strain SM10 λ pir and was then conjugally transferred into wild type _S. dysenteriae_ 1 strain SC500. Matings were performed on cellophane membranes, selection was obtained by plating on M9 minimal medium

35 supplemented with thiamine, methionine, tryptophan and

nicotinic acid at a concentration of 10 μg/ml each, 0.2% glucose and 50 μg/ml spectinomycin. Colonies growing on selective medium were purified and identified as S. dysenteriae 1 by agglutination with a specific rabbit antiserum (Diagnostics Pasteur).

Allelic exchange between the wild-type chromosomal Shiga-toxin gene and the in vitro mutagenized gene of Shiga-toxin was shown by colony blot immunoassay, using the monoclonal antibody 13C4 to detect S. dysenteriae 1 cells expressing a Tox- phenotype.

The presence of the Tox⁻ modification in the genomes of the S. dysenteriae 1 cells was verified with a probe made from the 655 bp HindIII-HincII fragment containing part of the A subunit gene and the entire B subunit gene from the 4.2 kb EcoRI fragment, described above, containing the entire Shiga-toxin operon. The 2 kb HindIII fragment, described above, containing the Ω interposon, was also used as a probe (37). The DNA fragments, used as the probes, were labeled by nick-translation (39) with ^{32}p-labeled 5'-dCTP (Amersham). Total DNA was prepared from two Tox- clones and analyzed by hybridization with the Shiga-toxin probe and the Ω probe. The DNA fragments were transferred from agarose gels to nitrocellulose filters (Schleicher and Schüll) by the method of Southern (53). Hybridization was carried out at 65°C overnight, and washing was done at 65°C in 6xSSC. The probes showed that the 4.2 kb EcoRI fragment from S. dysenteriae 1 containing the toxin genes had been replaced in the Tox- mutants by the 6.2 kb fragment, which hybridized with both probes. This result showed that the flanking regions on each side of the mutagenized toxin gene in pHS7203 had recombined with their counterparts in the SC500 genome, thus replacing the wild-type A subunit gene by the mutated gene.

One of these Tox- clones, SC501, was selected for further study, and clone SC501 was deposited with the Centre Nationale de Cultures de Microorganismes of Institut Pasteur, Paris, France, under accession no. I-774, on June 30, 1988.

Example 3 -- Assays of cytotoxicity, growth within HeLa cells, macrophage detachment and toxicity in Rabbit ileal loop and in monkey

SC500 and SC501, as well as their non-invasive derivatives SC502 and SC503 respectively (obtained by the spontaneous-cure (i.e., loss) of their large virulence plasmid pHS7200 which is necessary for invasion of cells), were grown for 48 hours in 200 ml of iron-depleted medium. Glassware was pretreated with 6N HCl and rinsed extensively with iron free H_2O. The medium contained M9 salts supplemented with 15 $\mu g/ml$ $CaCl_2$, 5mg/ml casamino-acids, 2mg/ml glucose, 50 $\mu g/ml$ thiamine, 20 $\mu g/ml$ L-tryptophane, 10 $\mu g/ml$ nicotinic acid and 150 $\mu g/ml$ human transferrin (Sigma). The bacteria were washed twice in saline and resuspended in 3 ml of PBS. Lysozyme was added at a final concentration of 0.2 mg/ml. After a 30 minute-incubation at room temperature (25°C), 30 μl EDTA 0.5 M pH8 was added, and the cells were transferred to an ice bath and sonicated. Sonic extracts were filter-sterilized and kept frozen at -20°C. Filter sterilized culture supernatants and bacterial extracts were assayed for cytotoxicity on HeLa cells grown in minimal essential medium with Earle's salts and N-glutamine (Gibco, Paisley, Scotland, UK) supplemented with 10% foetal calf serum (Gibco). Serial dilutions were made in cell culture medium (100 μl) in a microtitier plate. Each well was inoculated with 2 x 10⁴ cells in 100 μl. Plates were then incubated at 37°C in 5% CO_2 for 24 hours. Neutralization assays were performed both with a

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rabbit polyclonal serum and the 13C4 monoclonal antibody. Plates were examined under light phase microscopy, then stained with Giemsa. Cytotoxicity was calculated as the cytotoxic dose 50% (CD50) per mg of protein of the extract.

Multiplication of bacteria within HeLa cells was assayed (46). Non-confluent monolayers of HeLa cells in 35 mm plastic tissue culture dishes (Becton Dickinson Labware, Oxnard, CA, USA) were inoculated with bacteria, resuspended in 2 ml of minimum essential medium ("MEM", Gibco) at a multiple of infection ("MDI") of 100, centrifuged for 10 minutes at 2,200 x g and incubated for 30 minutes at 37°C to allow entry. Plates were then washed three times with Earle's Balanced Salt Solution ("EBBS", Gibco) and covered with 2 ml of MEM with gentamicin (25 μg/ml). This was defined as time 0 (To). After one hour of incubation at 37°C, preparations were washed again, with EBSS and covered with 2 ml of MEM without antibiotic (T1). Incubation was continued for three more hours (T1-T4). Two plates were removed every hour. One plate was washed three times with EBSS and Giemsa stained to calculate the percentage of infected HeLa cells. The other was washed five times with EBSS to eliminate viable extracellular bacteria. Cells were trypsinized, counted and lysed at 0.5% sodium deoxycholate in distilled water. Dilutions were plated onto Trypticase Soy Agar. The average number of bacteria per infected HeLa cell was calculated. Experiments were repeated four times. Intracellular growth curves were drawn and the slope at exponentional phase was calculated.

Assay for macrophage detachment and killing was performed (4) using J774 macrophages (52) maintained in RPMI 1640 (Flow Laboratories Inc., McLean, VA, USA) supplemented with complement-inactivated foetal calf

serum (Gibco) and 2 mM glutamine (Gibco). Eighteen hours before infection, 7×10^5 macrophages in 35 mm plastic tissue culture dishes (Becton Dickinson Labware) were labeled in a culture medium containing 0.5 μCi of [^{3}H] uridine per ml (Amersham). Cells were washed three times with EBSS before addition of 1 ml of the bacterial suspension in RPMI 1640 at a MOI of 100. Infection was performed for one hour at 37°C in 5% CO_2. Monolayers were then washed three times with EBSS (To) and covered for one hour at 37°C in 5% CO_2 with 2 ml of RPMI supplemented with 2 mM glutamine and gentamicin 25 μg/ml (T1). Plates were then washed three times with EBSS and incubated in 5% CO_2 for 3 more hours (T1-T4) at 37°C in RPMI glucose without gentamicin. Two plates were removed every hour, cultures were washed three times with EBSS and the percentage of non viable macrophages among cells that still adhered to the plastic surface was determined by trypan blue staining. The percentage of residual macrophages was then determined by measuring the amount of radioactivity remaining in the dish. Adherent cells were lysed with 1 ml of 0.5% sodium deoxycholate in distilled water and 100 μl of this lysate was precipitated and counted (4).

Rabbit ligated ileal loops of 10 cm were prepared in rabbits of ca. 2 kg which were anesthesized with 0.5 ml/kg of 6% sodium pentobarbital. Inocula of 10^7 and 10^9 CFU in 1 ml of Trypticase Soy Broth were tested. Rabbits were sacrificed 18 hours later. Fluid accumulation within loops was recorded, and the volume-to-length ration ("V/L") was calculated. Portions of infected loops were fixed in 10% buffered formalin. Specimens were processed by standard procedures and stained with hematoxylin-eosin-safranin.

Eight rhesus monkeys weighing 3.5 to 4.5 kg were injected intramascularly with 50 mg of ketamine

chlorhydrate (Imalgene 500, Rhône Mérieux, Lyon, France).
Each animal was inoculated intragastrically with 1.5 x
10^{11} of SC500 and SC501 microorganisms resuspended in 20
ml of Trypticase Soy Broth and 14 g/l sodium bicarbonate
(50/50). Plating of the inoculum on Congo-red agar
indicated that less than 1% of the bacteria in the
inoculum had lost their invasive property (26). Stools
were examined daily for diarrhea, presence of pus, mucus
and blood. Intensity of each of these symptoms was graded
from 0 to 3+ every day. For each animal, the severity of
a given symptom was expressed as an index which
represented a sum of the accumulated "+" for each
symptom. Immediate autopsy was performed in monkeys who
died of fulminant dysentery. Species ware processed as
described above for rabbit tissues.

RESULTS

SM10 λ pir (pHS7203) was noncytotoxic in the
cytotoxicity assay. After conjugative transfer of pHS7203
into S. dysenteriae, clones that displayed the Amp^s Spc^R
phenotype were tested in the colony immunoblot assay.
Five per cent displayed a Tox⁻ phenotype. SC501 showed a
cytotoxicity of 347 CD50/mg of protein, which was the
same order of magnitude as that of well-known E. coli K12
(412 CD50/mg). Residual cytotoxicity from SC501 could not
be neutralized by an anti-Shiga-toxin polyclonal serum.

The presence of the Tox- mutation in strain SC501
did not significantly alter its capacity to grow
intracellularly within HeLa cells since its rate of
exponential growth, expressed in generations/hour, was
2.6 ±0.7 compared to 2.5 ± 0.6 for wild-type strain
SC500. In addition, no significant difference could be
observed in the efficiency of rapid killing of J774
macrophages by SC500 and SC501. Both cell detachment and
appearance of Trypan Blue positive cells progressed at

similar rates over four hours, thus indicating that Shiga-toxin released within infected cells neither significantly affected the rate of intracellular growth nor increased rapid killing of host cells.

The effect of the Inv⁻ and Tox⁻ mutations on the pathogenicity of S. dysenteriae 1 in the rabbit ligated loop model was determined by the effect on fluid production within loops. Mean and standard deviations were computed from the results obtained in six loops for each strain at either of the two inocula (i.e., 10^9 and 10^7 CFU). For invasive strains (i.e., SC500, Inv⁺, Tox⁺ and SC501, Inv⁺, Tox⁻) at both inocula, the lack of Shiga-toxin production decreased fluid accumulation, but the difference was not statistically significant, indicating that invasion and subsequent inflammation are primarily responsible for fluid accumulation. For non-invasive strains (i.e., SC503, Tox⁺ and SC502, Tox⁻) a striking difference was observed since only the strain producing Shiga-toxin elicited fluid accumulation. This indicated that, in the rabbit model, Shiga-toxin is the only enterotoxin of S. dysenteriae 1, whatever the role this enterotoxin may play in the course of the disease. Histopathological studies showed severe lesions including abscesses and ulcerations destroying numerous villi at both inocula either with SC500 or SC501. In general, lesions were more severe in loops infected with the wild-type strain, but the observation that the difference was minor indicated that invasion was the major factor of pathogenecity.

Loops infected with SC502, the non-invasive Tox⁺ strain, were severely alterated with swelling and shortening of the villi, oedema and inflammation of the lamina propria, alterations of epithelial cells with large amounts of mucus shed from goblet cells and areas of killed enterocytes with pycnotic nuclei. However, the

most striking feature was hemorrhages throughout the epithelial layer.

The effect of the Tox⁻ mutation on the pathogenicity of S. dysenteriae 1 was shown in monkeys. Two animals died of fulminant dysentery at day 4 in both the group injected with SC500 and the group injected with SC501, each group thus indicating that Shiga-toxin was not required for lethal dysentery. No significant differences could be observed in the volume of diarrheic stools and the amount of pus and mucus, although the latter were difficult to quantify with precision. On the other hand, the presence of blood was a constant characteristic of abnormal stools in animals infected with SC500 whereas only one animal infected with SC501 showed transcient presence of a slight amount of blood. Autopsies performed immediately after the death of the animals showed obvious differences in the colonic peritoneal mesothelium which was particularly apparent on the surface of the sigmoïd on which patchy hemorrhagic areas could be observed only in the case of animals infected with SC500. On the average, the number and severity of abscesses was similar, but purulent necrosis of the mucosa with destruction in Lieberkühn glands was only observed, in some areas, in animals infected with SC500. Inflammatory infiltration of the chorion, submucosal tissues and peritoneum was also more severe in these animals. In addition, the inflammatory infiltrate of the peritoneal mesothelium which was characteristic of animals infected with SC500 as compared to SC501, was predominantly perivascular thus confirming the gross examination which suggested the presence of a severe peritoneal vasculitis. However, the most striking difference was observed at the level of the capillary circulation within the interglandular chorion. Monkeys infected with SC500 showed hemorrhages disrupting the

structure of the upper part of the mucosa. Erythrocytes could be observed being released into the intestinal luman through microabscesses which caused local interruption of the epithelial lining. These hemorrhages were obviously due to destruction of the capillary loops. On the other hand, monkeys infected with SC501 showed dilatation of the capillary loop but no disruption. White blood cell counts performed before and at day 3 after infection showed: at day 0, no significant difference in polymorpho nuclear cell ("PMN") counts, and myelemia was absent; and at day 3, the drop in blood PMN and the level of myelemia were each more pronounced in monkeys infected by SC500.

CONCLUSIONS

Circumstantial evidence in humans supports the hypothesis that Shiga-toxin is a true virulence factor. Volunteers fed strain 725, an invasive, low-toxin producing, chlorate-resistant mutant of S. dysenteriae 1, showed less severe symptoms than those fed the wild-type strain M131 (25). Patients experiencing natural infection usually develop more severe symptoms including HUS when infected with S. dysenteriae 1 than with other Shigella serotypes (7). They rapidly develop toxin-neutralizing antibodies (18).

The Tox⁻ mutant of S. dysenteriae 1, SC501, has been shown to produce a residual amount of cytotoxin similar to E. coli K12. This mutant has been used to study the role of this Shiga-toxin in the virulence of S. dysenteriae 1. Cellular assays and more definitive animal models have been used.

Assays using HeLa cells and J774 macrophages in monolayers have shown that secretion of Shiga-toxin did not affect the rate of exponential growth within infected cells as suggested for SLT in S. flexneri in a previous

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study (46). These results were in agreement with the observation that two other low toxin producer mutants (25, 48) as well as the SC501 mutant do not affect keratoconjuctivitis (49) which is known to correlate with the capacity of bacteria to multiply within an epithelium (35). As also suggested previously (4, 12), no correlation could be observed between Shiga-toxin production and early killing of host cells. Although such data need confirmation in assays that would more closely mimic the actual infection, they certainly indicate that Shiga-toxin does not play a major role at the intracellular stage of infection. Invasion appears to trigger early metabolic events which mediate killing of host cells (47) more rapidly than the slow acting process of Shiga-toxin (12).

Infection of rabbit ligated intestinal loops demonstrated only slight differences in the severity of mucosal lesions after 18 hours with both the SC500 and SC 501 inocula. However, the duration of exposure and closing of loops may mask the effect of cytotoxin production and make invasion the primary event. Results concerning enterotoxicity were more difficult to analyze in the case of invasive bacteria since the amount of fluid produced, although lower at both inocula for the Tox⁻ mutant, was not significantly different from that elicited by the wild-type strain. This indicated that invasion of tissues is sufficient to block the reabsorbative functions of the epithelium. On the other hand, the striking difference observed between non-invasive Tox⁺ and Tox⁻ mutants indicates that, within the limits of sensitivity of the rabbit model, Shiga-toxin is the only enterotoxin of S. dysenteriae 1. This is in agreement with previous studies (16, 17, 33). However, when observing fluid production by Inv⁺ and Inv⁻ mutants, the nature of the fluid produced varies according to the

infecting strain. Invasive strains elicit production of a viscous, mucopurulent, sometimes bloody liquid which probably reflects the extent of abscesses ulcerated within the lumen   regardless of the amount of <u>Shiga</u>-toxin produced, whereas non-invasive, Tox* strains produce a watery, sometimes bloody, liquid which is more a reflection of enterotoxicity and cytotoxicity. Histopathological studies of tissue samples from loops infected with SC502, the Inv*, Tox* mutant, showed an important inflammatory infiltrate of the lamina propria and major alterations predominantly at the tip of shortened villi. This confirmed the cytotoxicity of <u>Shiga</u>-toxin on enterocytes <u>in</u> <u>vivo</u> (27). However, the most striking feature was infiltration of the epithelial lining by erythrocytes which were shed into the lumen along with important amounts of mucus. This observation, which suggested that major vascular alterations had occurred within the lamina propria, was subsequently confirmed in the monkey model.

Intragastric inoculation of SC500 and SC501 in macaque monkeys demonstrated that lethal fulminant dysentery could occur regardless of <u>Shiga</u>-toxin production. No significant difference was observed in the amount of diarrhea, pus and mucus in stolls. Absence of watery diarrhea and equal amount of stool were not consistent with previous studies suggesting increased jejunal secretion bu <u>Shiga</u>-toxin (41). The only striking difference was the presence of blood in dysenteric stools of animals infected with the wild type strain. A recent paper reported that, among patients presenting shigellosis, those who eliminated strains of higher cytotoxicity were more likely to present blood in their stools (36). Histopathological observations confirmed the presence of vascular damages which appeared particularly characteristic in the sigmoid since monkeys infected with

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the wild type strain showed total destruction of the capillary loops within the chorion whereas the vascular system of animals infected with the Tox⁻ mutant showed turgescent but mostly intact vessels. This certainly explains the presence of bloody stools in the former group. In addition, observation of the peritoneal mesothelium showed oedema and severe inflammatory vasculitis. Thus, release of Shiga-toxin by invading bacteria within the tissues may locally enhance severity of the mucosal lesion by evoking local ischemia through destruction of the chorion blood flow and alterations of the peritoneal as well as possibly mesenteric circulation. This effect appears to be local or loco-regional since observation of kidney tissues did not show evidence of capillary vasculitis at this stage of the disease (data not shown). Such vascular alterations may be consistent with observations in hemorrhagic colitis due to E. Coli 0157:H7 (40) in which a radiologic aspect of ischemic colitis has been described (34). These strains produce high levels of SLT1 (31) which has a direct cytopathic effect on dividing endothelial cells (15).

Another difference observed between animals infected with Tox⁺ and Tox⁻ strains was the severity of mucosal inflammation and subsequent abscesses. In many areas of the sigmoid and transverse colons, lesions appeared of similar intensity, but only animals infected with SC500 showed areas with impressive purulent destruction of mucosal tissues.

Higher intensity of the purulent exsudate was reflected in a more dramatic drop of blood PMN with consecutive myelemia at day three of infection. It is believed that, in addition to the marrow and vascular compartments, a third PMN compartment is opened at the colonic level during shigellosis. Shiga-toxin is expected

to increase the number of PMN entrapped within this new compartment through vascular alterations which increase diapedesis as well as direct release of PMN within mucosal tissues. This would account for the rapid and severe granulocytopenia observed in animals infected by the wild type strain and for subsequent higher myelemia which may be an equivalent of the leukemoid reaction sometimes observed in the course of severe shigellosis. Such a model does not postulate a systemic effect of Shiga-toxin.

The foregoing results thus suggest that Shiga-toxin plays a limited role when released intracellularly within epithelial and phagocytic cells. However, Shiga-toxin released within infected tissues appears to act predominantly through intestinal vascular damage.

Example 4

Using the procedure of Example 2, SC501 is genetically engineered by in vitro mutagenesis of its operon coding for enterochelin. The suicide plasmid vector pJM703.1, that is utilized, contains the enterochelin operon of S. dysenteriae 1, with each of its ent F, Fep E, Fep C and Fep D subunit genes mutagenized with an interposon which codes for resistance to the herbicide Biolafos and a suitable promoter for the herbicide resistance gene. The resulting clone, SC504, is Tox⁻ and enterochelin⁻ ("Ent⁻").

Example 5

Using the procedure of Example 2, SC504 is genetically engineered by in vitro mutagenesis of its ics A gene. The suicide plasmid vector pJM703.1, that is used, contains the ics A gene of S. flexneri (60, 61), which has been mutagenized with an interposon. The resulting clone, SC505, is Tox⁻, Ent⁻ and ics A⁻ and can be used in making a vaccine against S. dysenteriae 1.

Example 6

Using the procedure of Example 2, a wild type S. flexneri is genetically engineered by in vitro mutagenesis of its gene coding for aerobactin and its ics A gene. The suicide plasmid vector, that is used, contains the aerobactin and ics A genes of S. flexneri which have each been mutagenized with an interposon. The resulting clone, SC506, is aerobactin⁻ and ics A⁻ and can be used in making a vaccine against S. flexneri.

Example 7

Using the procedure of Examples 1, 2 and 4, a 400 basepair Bal31 deletion is made, starting from the unique HpaI site, inside the A subunit gene of the Shiga-toxin operon in a DNA fragment from S. dysenteriae 1 in strain SC500. The resulting fragment is religated with a 257 basepair fragment containing the P1 promoter of pBR322, thus allowing high expression of the B subunit protein. This fragment, containing the mutagenized toxin A gene, is cloned into a conditional suicide vector which contains a replication of origin under the control of the E. coli lac promoter and a kanamycin resistance gene. In S. dysenteriae 1, this vector will replicate only if IPTG is present in the culture medium. A mercury-resistance cartridge (65) is inserted upstream from the mutagenized A subunit gene. The resulting plasmid is transformed into the wild type S. dysenteriae 1 strain SC500 in the presence of IPTG. Colonies of the resulting Shigella clone are Hg and kanamycin resistant. They are allowed to grow for many generations in the absence of IPTG. The cultures are then screened for the presence of Hg-resistant kanamycin-sensitive clones. Three clones are isolated and further characterized. Southern blots show that they no longer hybridize with an A subunit gene internal probe but still produce high amounts of B

subunit protein, as detected by monoclonal antibody analysis, and they no longer are cytotoxic.

Using the same procedure, this ToxA⁻ clone is genetically engineered by _in vitro_ mutagenesis of its operon coding for enterochelin. The suicide plasmid vector, that is utilized, contains the enterochelin operon of _E. coli_ (66), with each of its ent F, Fep E, Fep C and Fep D subunit genes having a significant deletion at a restriction site, into which is inserted a fragment that codes for resistance to arsenite (62) and a suitable promoter for the arsenite-resistance gene. The resulting clone is Tox A⁻ and Ent⁻.

Using the same procedure, this Tox A⁻ and Ent⁻ clone is genetically engineered by _in vitro_ mutagenesis of its _ics_ A gene. The suicide plasmid vector, used, contains the _ics_ A gene of _S. flexneri_ (60, 61), that has a significant deletion at a restriction site, into which is inserted a fragment coding for resistance to cadmium (63, 64) and a suitable promoter for the cadmium-resistance gene. The resulting Tox A⁻, Ent⁻, _ics_ A⁻ _S. dysenteriae_ 1 clone is characterized by a substantially reduced invasiveness, which renders it suitable for making a vaccine for humans against _S. dysenteriae_ 1.

It is believed that this invention and many of its attendant advantages will be understood from its description above, and it will be apparent that various modifications can be made in the method and vaccine described above without departing from the spirit and scope of the invention or sacrificing all of its material advantages, the embodiments described above being merely preferred embodiments.

The references, referred to above, are as follows.

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Table 1 : Strains, plasmids, phages and their relevant characteristics

Strain	Species	Genotype	Plasmid/ phage	Relevant characteristics
SC 500	S. dysenteriae 1	thi, nad, trp, met	pHS7200	Invasion of HeLa cells
SC 501	S. dysenteriae 1	thi, nad, trp, met, tox, spc^r	pHS7200	Invasion of HeLa cells
SC 502	S. dysenteriae 1	thi, nad, trp, met	-	-
SC 503	S. dysenteriae 1	thi, nad, trp, met, tox, spc^r	-	-
Y 1089	E. coli	ΔlacU169 proA⁺ Δlon araD139 strA hfl Δ150[chr::Tn10]	pMC9	Ap^r, pBR322-lac i^q
			λGT11	lac5Δ (shindIIIΔ2-3) srIλ3⁻ cI857 srIλ4⁻ nin5 srIλ5⁻ sam100
Y 1090	E. coli	ΔlacU169 proA⁺ Δlon araD139 strA supF[trpC22::Tn10]	pMC9	Ap^r, pBR322-lac i^q
JM 83	E. coli	F⁻, araΔlac-pro strA thi, phi80dlacZ ΔM15	pUC8	Ap^r, cloning vehicle
			pHS7201	Ap^r, Shiga toxin genes subcloned in pUC8
			pHS7202	Ap^r Spc^r Ω is inserted at the BpaI site of pHS6001
			pHP45	Ap^r Spc^r contains the Ω element
SM10λpir	E. coli	recA, RP4-2 TC::Mu Km^r thi, chr, leu, suIII	λpir	contains the pir function from R6K replication origin
			pJM703-1	Suicide cloning vector Ap^r, can be mobilised in SM10λpir
			pHS7203	Mutagenized toxin genes cloned in pJM703-1 Ap^r Sp^r
HB101	E. coli	RB⁻, MB⁻, recA, supE44 (su2)lacY, leuB6 proA2 thi-1 Sm^r	-	-

C L A I M S

1. A method for modifying a wild strain of an entero-invasive _Shigella_ to produce a modified strain of _Shigella_ that can be used for making a vaccine against the wild strain of _Shigella_ characterized by the step of transforming the genome of the wild strain of _Shigella_ so that it cannot substantially invade cells of a host and cannot spread substantially within infected cells and from infected to uninfected cells of the host and cannot produce toxins which will kill substantial numbers of the host's infected, as well as uninfected, cells.

2. The method of claim 1 in which the genome of the wild strain of _Shigella_ is modified so that a first gene, coding for a protein necessary for the wild strain of _Shigella_ to invade cells, as well as tissues, of the host, and a second gene, coding for a protein necessary for the wild strain of _Shigella_ to spread within infected cells and between infected and uninfected cells of the host, are wholly or partly removed or permanently inactivated.

3. The method of claim 2 in which the _Shigella_ is an _S. flexneri_ and the first gene codes for the production or use of aerobactin by the _S. flexneri_.

4. The method of claim 3 in which the second gene codes for intra-intercellular spread.

5. The method of claim 2 in which the _Shigella_ is an _S. dysenteriae_ 1, the genome of which is modified so that a third gene, coding for the production or use of _Shiga-toxin_ by the _S. dysenteriae_ 1, is wholly or partly removed or permanently inactivated.

6. The method of claim 5 in which the first gene of the _S. dysenteriae_ 1 codes for the production or use of enterochelin by the _S. dysenteriae_ 1.

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7. The method of claim 6 in which the second gene codes for intra-intercellular spread.

8. The method of claim 6 in which the first gene comprises the ent F, Fep E, Fep C and Fep D subunit genes of the enterochelin operon of the S. dysenteriae 1.

9. The method of anyone of claims 5-8 in which the first, second and third genes are mutagenized.

10. The method of anyone of claims 2-9 in which one or more of the genes are inactivated by allelic exchange with one or more in vitro mutagenized genes, especially mutagenized genes from which significant portions have been deleted and particularly mutagenized genes into which marker genes have been inserted.

11. A Shigella which has been modified by the method of anyone of claims 1-10 or is a descendant thereof.

12. A vaccine which has been made from the modified Shigella of claim 11.

13. A Shigella which is Shiga-toxin⁻, particularly Shiga-toxin A⁻.

A B S T R A C T

A method for modifying a wild strain of an entero-
invasive <u>Shigella</u> to produce a modified strain of
<u>Shigella</u> that can be used for making a vaccine against
the wild strain of <u>Shigella</u>. The genome of the wild
strain of <u>Shigella</u> is transformed so that it cannot
substantially invade cells of a human host and cannot
spread substantially within infected cells and from
infected to uninfected cells of the host and cannot
produce toxins which will kill substantial numbers of the
host's infected, as well as uninfected, cells. A first
gene of the wild strain of <u>Shigella</u>, coding for a protein
necessary for the <u>Shigella</u> to invade cells of the host,
and a second gene, coding for a protein necessary for the
<u>Shigella</u> to spread within infected cells and between the
infected and uninfected cells of the host, are
mutagenized.

24. A method for modifying a wild strain of an enteroinvasive *Shigella* to produce a modified strain of *Shigella* that can not spread substantially within infected cells of a host and can not spread substantially from infected to uninfected cells of the host, for use in making a vaccine against the wild strain of *Shigella*, the method comprising inactivating an *ics*A gene of the wild strain of *Shigella*, other than only by inactivation by means of a transposon inserted into the gene, to thereby provide a modified strain of *Shigella* that can not spread substantially within infected cells of the host and can not spread substantially from infected to uninfected cells of the host.

25. The method of claim 24, wherein the modified strain of *Shigella* also can not substantially invade cells of the host, the method further comprising inactivating an aerobactin or enterochelin gene of the wild strain of *Shigella*, other than only by inactivation by means of a transposon inserted into the gene, to thereby provide a modified strain of *Shigella* that can not spread substantially within infected cells of the host, can not spread substantially from infected to uninfected cells of the host, and can not substantially invade cells of the host.

26. The method of claim 25, wherein the modified strain of *Shigella* also can not produce toxins that kill a substantial number of the host's cells, the method further comprising inactivating a *Shiga*-toxin gene of the wild strain of *Shigella*, other than only by inactivation by means of a transposon inserted into the gene, to thereby provide a

modified strain of *Shigella* that can not spread substantially within infected cells of the host, can not spread substantially from infected to uninfected cells of the host, can not substantially invade cells of the host, and can not produce toxins that kill a substantial number of the host's cells.

27. The method of any of claims 24-26, wherein said *Shigella* is *S. flexneri*.

28. The method of any of claims 24-26, wherein said *Shigella* is *S. dysenteriae* 1.

29. The method of claim 28, wherein one or more of the ent F, Fep E, Fep C, and Fep D subunit genes of the enterochelin operon of *S. dysenteriae* 1 are modified.

30. The method of claim 26, wherein the *Shiga*-toxin gene is the *Shiga*-toxin A gene.

32. The method of any of claims 24-26, wherein one or more of said inactivated genes are inactivated genes from which at least one nucleotide sequence has been deleted.

33. The method of any of claims 24-26, wherein one or more of said inactivated genes are inactivated genes into which at least one nucleotide sequence has been inserted.

34. The method of claim 33, wherein a marker gene is inserted into one or more of said inactivated genes.

35. The method of claim 26, further comprising isolating said modified strain of *Shigella* from said wild strain of *Shigella*.

36. A modified *Shigella* for use in making a vaccine against a wild strain of *Shigella*, the modified *Shigella* comprising:

(a)   an inactivated icsA gene, inactivated other than only by means of a transposon inserted into the gene; and

(b)   an inactivated aerobactin or enterochelin gene, inactivated other than only by means of a transposon inserted into the gene;

wherein the modified *Shigella* can not spread substantially within infected cells of the host, can not spread substantially from infected to uninfected cells of the host, and can not substantially invade cells of the host.

37. The *Shigella* of claim 36, further comprising an inactivated *Shiga*-toxin gene, inactivated other than only by means of a transposon inserted into the gene;

wherein the modified *Shigella* can not spread substantially within infected cells of the host, can not spread substantially from infected to uninfected cells of the host, can not substantially invade cells of the host, and can not produce toxins that kill a substantial number of the host's cells.

39. The *Shigella* of claim 37, wherein the *Shiga*-toxin gene is *Shiga*-toxin A.

40. The *Shigella* of claim 36 or 37, wherein said *Shigella* is *S. dysenteriae* 1 or *S. flexneri*.

41. The *Shigella* of claim 36 or 37, comprising inactivated ent F, Fep E, Fep C, or Fep D subunit genes of the enterochelin operon.

43. The *Shigella* of claim 36 or 37, wherein one or more of said inactivated genes are inactivated genes from which at least one nucleotide sequence has been deleted.

44. The *Shigella* of claim 36 or 37, wherein one or more of said inactivated genes are inactivated genes into which at least one nucleotide sequence has been inserted.

45. The *Shigella* of claim 44, wherein a marker gene is inserted into one or more of said inactivated genes.

46. A vaccine comprising the *Shigella* of claim 36 or 37 and a pharmaceutically acceptable vehicle.

47. The method of any of claims 24-26, wherein a marker gene is inserted into each inactivated gene.

48. The *Shigella* of claim 36 or 37, wherein a marker gene is inserted into each inactivated gene.

49. A vaccine comprising the *Shigella* of claim 48 and a pharmaceutically acceptable vehicle.

50. The method of claim 24, wherein said inactivation of said *icsA* gene comprises allelic exchange with a mutagenized *icsA* gene that has been mutagenized *in vitro*.

51. The method of claim 25, wherein said inactivation of said *icsA* gene comprises allelic exchange with a mutagenized *icsA* gene that has been mutagenized *in vitro*, and wherein said inactivation of said aerobactin or enterochelin gene comprises allelic exchange with a mutagenized aerobactin or enterochelin gene that has been mutagenized *in vitro*.

52. The method of claim 26, wherein said inactivation of said *icsA* gene comprises allelic exchange with a mutagenized *icsA* gene that has been mutagenized *in vitro*, wherein said inactivation of said aerobactin or enterochelin gene comprises allelic exchange with a mutagenized aerobactin or enterochelin gene that has been

mutagenized *in vitro*, and wherein said inactivation of said *Shiga*-toxin gene comprises allelic exchange with a mutagenized *Shiga*-toxin gene that has been mutagenized *in vitro*.

53. The method of any of claims 50-52, wherein a marker gene is inserted into one or more of said mutagenized genes.

54. A modified *Shigella* for use in making a vaccine against a wild strain of *Shigella*, the modified *Shigella* comprising:

(a) an inactivated *icsA* gene, inactivated by allelic exchange with a mutagenized *icsA* gene that has been mutagenized *in vitro*, wherein said mutagenesis is other than only by means of a transposon inserted into the gene; and

(b) an inactivated aerobactin or enterochelin gene, inactivated by allelic exchange with a mutagenized aerobactin or enterochelin gene that has been mutagenized *in vitro*, wherein said mutagenesis is other than only by means of a transposon inserted into the gene;

wherein the modified *Shigella* can not spread substantially within infected cells of the host, can not spread substantially from infected to uninfected cells of the host, and can not substantially invade cells of the host.

55. The *Shigella* of claim 54, further comprising an inactivated *Shiga*-toxin gene, inactivated by allelic exchange with a mutagenized *Shiga*-toxin gene that has been

mutagenized *in vitro*, wherein said mutagenesis is other than only by means of a transposon inserted into the gene;

wherein the modified *Shigella* can not spread substantially within infected cells of the host, can not spread substantially from infected to uninfected cells of the host, can not substantially invade cells of the host, and can not produce toxins that kill a substantial number of the host's cells.

56. The *Shigella* of claim 54 or 55, wherein a marker gene is inserted into one or more of said mutagenized genes.

57. A vaccine comprising the *Shigella* of claim 54 or 55 and a pharmaceutically acceptable vehicle.

74. A modified *Shigella* for use in making a vaccine against a wild strain of *Shigella*, the modified *Shigella* comprising an inactivated *icsA* gene, inactivated other than only by means of a transposon inserted into the gene;

wherein the modified *Shigella* can not spread substantially within infected cells of the host and can not spread substantially from infected to uninfected cells of the host.

75. The *Shigella* of claim 74, wherein at least one nucleotide sequence has been deleted from said inactivated *icsA* gene.

76. The *Shigella* of claim 74, wherein at least one nucleotide sequence has been inserted into said inactivated *icsA* gene.

77. The *Shigella* of claim 76, wherein a marker gene is inserted into said inactivated *icsA* gene.

78. A vaccine comprising the *Shigella* of claim 74 and a pharmaceutically acceptable vehicle.

79. A modified *Shigella* for use in making a vaccine against a wild strain of *Shigella*, the modified *Shigella* comprising an inactivated *icsA* gene, inactivated by allelic exchange with a mutagenized *icsA* gene that has been mutagenized *in vitro*, wherein said mutagenesis is other than only by means of a transposon inserted into the gene;

wherein the modified *Shigella* can not spread substantially within infected cells of the host and can not spread substantially from infected to uninfected cells of the host.

80. The *Shigella* of claim 79, wherein a marker gene is inserted into said mutagenized *icsA* gene.

81. A vaccine comprising the *Shigella* of claim 79 and a pharmaceutically acceptable vehicle.

A Genetic Determinant Required for Continuous Reinfection of Adjacent Cells on Large Plasmid in S. flexneri 2a

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Summary

We have identified a region (virG) on the 230 kb virulence plasmid of S. flexneri that is required for cell-to-cell spread of the bacterium. Tn5 insertions into this region result in avirulent mutants that can initially invade and multiply in epithelial cells, but tend to lose active movement and tend to localize within the cytoplasm, where they are gradually extinguished without infecting adjacent cells. The virG region was localized to within 4 kb and may contain a single cistron. Sequences hybridizing to this region were found in all intact virulence plasmids of Shigellae and enteroinvasive E. coli.

Introduction

Shigellae are among the invasive pathogens and provoke bacillary dysentery in human and monkey. The early essential step for the virulence of Shigellae is the invasion into colonic epithelial cells (LaBrec et al., 1964), followed by intracellular bacterial multiplication and reinfection of adjacent cells. Strains that lack any of these abilities are avirulent. At least three separate loci have been identified on the chromosome to be virulence determinants of Shigella flexneri (Sansonetti et al., 1983). In addition, a large plasmid (about 230 kb) is required for the invasion into colonic epithelial cells (Sansonetti et al., 1982). The plasmid is functionally common among four subgroups of Shigellae and enteroinvasive E. coli (Hale et al., 1983; Watanabe and Nakamura, 1985).

Laboratory assays for virulence in Shigellae have mainly been made by inducing conjunctivitis in guinea pigs (Sereny test; Sereny, 1955) or mice (mouse Sereny test; Murayama et al., 1986), and the invasion into cultured cells (Maurelli et al., 1984). The virulence of Shigellae is highly unstable in subculture or storage (Maurelli et al., 1984; Sansonetti et al., 1982; Sasakawa et al., 1986). Such avirulent derivatives score negatively in mouse Sereny tests (Ser) and are unable to invade cultured cells (Inv). These derivatives show either deletions of, or IS1 insertion into, the 230 kb plasmid, pMYSH6000 (Sasakawa et al., 1986). By analyzing these mutants, it became evident that there are at least two virulence determinants about 50 kb apart (Sasakawa et al., 1986; Sakai et al.,

1986). Subsequent studies by Tn5 insertion inactivation on pMYSH6000 revealed that a large region consisting of four contiguous SalI cleavage fragments, B, P, H, and D, and two separate fragments, F and G, are required for the Ser⁺ phenotype. All of these regions, except the SalI cleavage fragment, G (SalI-G), have been shown to be required for invasion into epithelial cells (Inv). Interestingly, the region on SalI-G was required only for Ser but not for Inv (Sasakawa et al., submitted).

In this communication, the genetic determinant of this region (abbreviated as virG, for virulence) on SalI-G is precisely defined, and the role that the product of this region plays in an essential step in the pathogenesis of bacillary dysentery is described.

Results

Microscopic Observation of Bacterial Invasion in Tissue Culture

We isolated 12 avirulent Tn5 insertion mutants in SalI-G of pMYSH6000, a large plasmid found in S. flexneri 2a YSH6000. All of them were apparently positive in the standard invasion test. However, we noted differences in the localization and movement of the wild-type virulent strain, YSH6000, and the avirulent SalI-G insertions, within MK cells. To assess the significance of this difference, the time course of invasion into MK cells was examined from time 0 to 18 hr. Up to 1 hr following bacterial infection, invasion of the SalI-G mutant, M94, was similar to that of YSH6000, and no difference was observed in the number of bacteria within MK cells (Figures 1A, 1B, 1E, and 1F). After 6 hr, YSH6000 showed free and active movement within host cell cytoplasm and reinfected adjacent cells (Figure 1C). However, the invading M94 bacteria, exhibited normal multiplication initially, but were localized within the cytoplasm and had assumed spherical shapes (Figure 1G). After 18 hr, the morphological differences between YSH6000 and M94 were more obvious. MK cells invaded by YSH6000 did not adhere to the culture plate, the number of MK cells was decreased, and apparently viable bacteria were scattered outside the cell cytoplasm. In the case of M94, intracytoplasmic bacteria that had been converted to spheres were extinguished, and MK cells had returned to their initial normal morphology. In summary, the avirulent SalI-G mutant can multiply in the epithelial cells in vitro but are extinguished before they can spread and infect adjacent cells.

The Process of Infection In Vivo as Revealed by Indirect Fluorescence and Histopathological Methods

To assess the difference in the ability of YSH6000 and M94 in invading keratoconjunctival epithelia, about 30 μl of the bacteria at a density of 10^{10} viable cells per ml was dripped into guinea pigs' eyes. Three hours or 24 hr after infection, bacterial antigens were detected by indirect immunofluorescence and were assayed for the ability to induce keratoconjunctivitis shigellosa and for the invasion
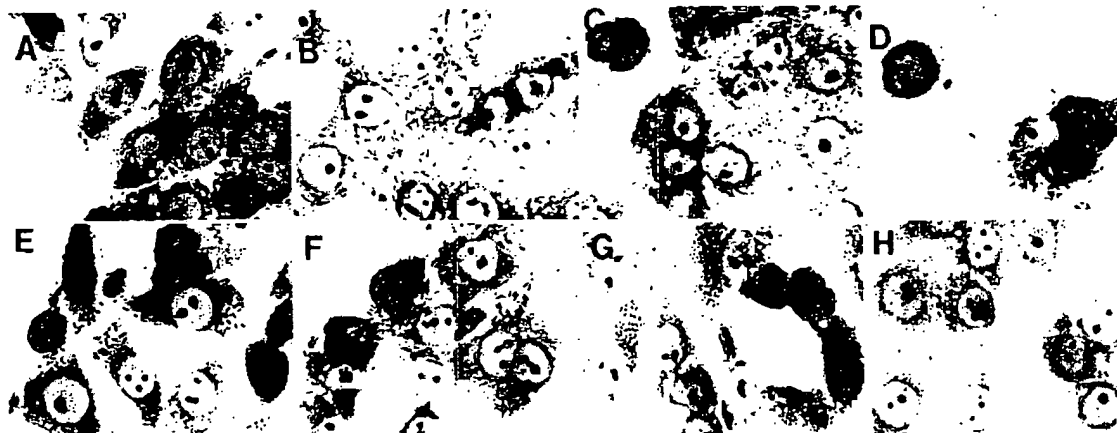
Figure 1. Time Course of Invasion into Tissue-Cultured MK Cells of the Virulent Wild Type, YSH6000, and Its Avirulent Tn5 Insertion (M94) into SalI-G of pMYSH6000

(A–D) YSH6000. (E–H) M94. Samples were taken at the indicated times, following bacterial infection for 40 min. (A and E) 0 hr. (B and F) 1 hr. (C and G) 6 hr. (D and H) 18 hr.
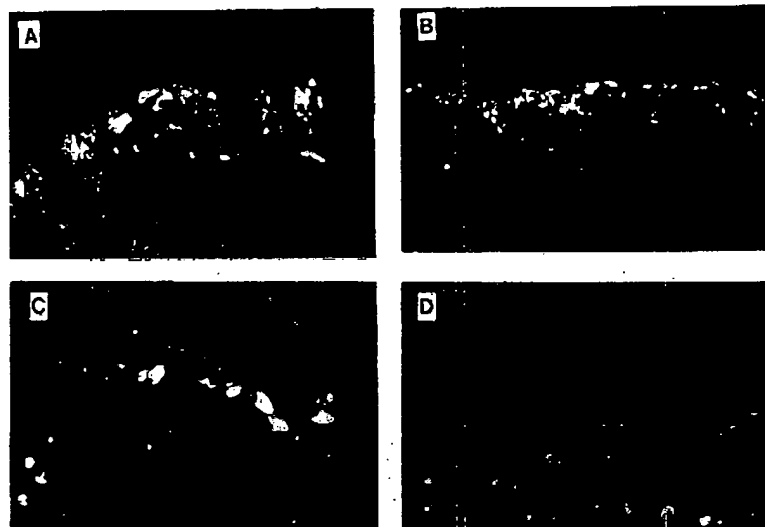

Figure 2. Indirect Immunofluorescence Analysis of the Epithelial Layers of Palpebral Conjunctiva, Fornix, and Cornea in Paraffin Sections

(A and B) YSH6000. (C and D) M94. (A and C) 3 hr; (B and D) 24 hr after infection.

of bacteria into keratoconjunctival epithelia. By macroscopic observation at 3 hr, no sign of keratoconjunctivitis was observed with either YSH6000 or M94 (data not shown). However, YSH6000 invaded the full layer of corneal epithelia (Figure 2A); M94 invaded only the single surface layer of the palpebra at 3 hr, and the bacterial morphology within the epithelia was spherical (Figure 2C). Epithelial degeneration was observed with YSH6000 but not with M94. At 24 hr after infection, YSH6000, but not M94, induced typical keratoconjunctivitis with desquamation due to degeneration of corneal epithelia, and consequently, only a single basal epithelial layer had remained, where bacteria could be seen (Figure 2B). In the case of M94, bacteria could not be seen in cornea and in palpebra that was not degenerated or desquamated (Figure 2D). These in vivo observations agree completely with in vitro observations.

Identification of the *virG* Region

By determining the fine map of the Tn5 insertion sites within SalI-G (Figure 3), the *virG* region responsible for virulence on SalI-G was identified. Since Tn5 has no EcoRI cleavage site, each plasmid DNA with a Tn5 insertion within SalI-G was digested with EcoRI and linked to the EcoRI site of pBR333 (Sasakawa et al., 1983) by shot-gun cloning and selecting for kanamycin resistance. Then, the Tn5 insertion site and direction were determined by digesting with EcoRI alone, EcoRI and SalI (double), and EcoRI, SalI, and BamHI (triple) (Figure 3). The Tn5 insertion sites of avirulent derivatives were located as a cluster within about 3 kb (Figure 4). Since the Tn5 insertion sites of the remaining virulent derivatives were located only to the right of this cluster, the left terminus of *virG* could not be determined by the fine map of the Tn5 insertion sites. The left terminus will be described below.
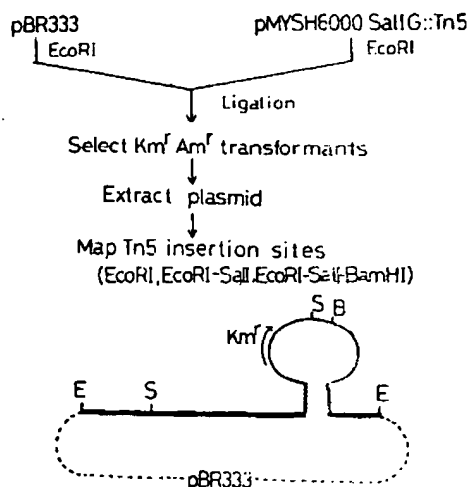
Virulence Determinant on S. flexneri Plasmid
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Figure 3. The Strategy for Fine Mapping of the Tn5 Insertion Sites
E, EcoRI; S, SaII; B, BamHI; thick solid line, derived from pMYSH6000; thin solid line, derived from the internal part of Tn5; wavy lines, IS50 parts of Tn5; broken line, pBR333 vector.
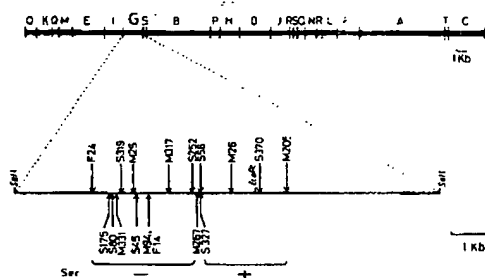

Figure 4. SaII Restriction Endonuclease Cleavage Map of pMYSH-6000 and the Tn5 Insertion Sites Located on the SaII Fragment of Virulent and Avirulent Derivatives

Upward and downward vertical arrows are the Tn5 insertion sites to the opposite direction. Ser, mouse Sereny tests; --, Sereny negative; +, Sereny positive.

To confirm that Tn5 insertions into SaII-G produced the avirulent phenotype because of insertional inactivation of the virulence gene(s) on SaII-G, the 6.1 kb SaII-EcoRI fragment of pMYSH6000 was cloned into pMY6003 to obtain pMYSH6601, which contains the above-mentioned 3 kb cluster. pMYSH6601 was transformed to 12 avirulent mutants, and the virulence of the transformants was examined by the mouse Sereny test. All of them were found to be converted to Ser+ (Table 1). Analysis of plasmid DNA of the transformants revealed the intactness and independent coexistence of each of the original Tn5 insertion derivatives and pMYSH6601. These observations show that the 6.1 kb SaII-EcoRI fragment in SaII-G contains the virG region, which is phenotypically expressing trans-acting product(s).

Analysis of the virG Region

To examine whether virG consists of a single cistron, complementation tests were made (Table 1). Plasmid DNAs from each of four avirulent mutants, F24, S252, S175, and

Table 1. Complementation Tests

virG::Tn5	Complementation by					
	None	pMYSH6601	F24	S252	S175	M267
F24	–	+	–	–	ND	–
S319	–	+	–	–	ND	ND
M25	–	+	–	–	ND	ND
M317	–	+	–	–	ND	ND
S252	–	+	–	–	ND	ND
S175	–	+	ND	ND	–	–
S80	–	+	ND	ND	–	–
M331	–	+	ND	ND	–	–
S45	–	+	ND	ND	–	–
M94	–	+	ND	ND	–	–
F14	–	+	ND	ND	–	–
M267	–	+	–	ND	–	–

(+) Positive reaction of mouse Sereny test.
(–) Negative reaction of mouse Sereny test.
ND, not determined.

M267, were isolated, digested with EcoRI, and cloned into the EcoRI site of pMY6003 by selecting for kanamycin resistance. The cloned plasmids contain the virG::Tn5 segment (see Figure 3). These recombinant plasmids were used to transform 12 avirulent mutants, and mouse Sereny tests were conducted with these transformants. All complementation tests among mutants were negative (Table 1). These results suggest that the virG region may contain a single cistron.

The 6.1 kb SaII–EcoRI fragment was employed to define the left end of the virG gene(s). Five insertion and deletion mutants were constructed and used to transform avirulent mutant M94, and the virulence of these transformants was examined by mouse Sereny test. An insertion mutant pMYSH6602, but not pMYSH6603, complemented the mutation of M94. These results and the Tn5 insertion site of F24 placed the left terminus between two EcoRV sites, indicating that the size of the virG gene(s) is 3 kb to 4 kb (Figure 5).

Distribution of the virG Region

Using the 1.2 kb HindIII–EcoRV fragment (Figure 5) as probe, Southern hybridization was performed against the large plasmids contained in each of the virulent strains S. dysenteriae, S. flexneri, S. boydii, S. sonnei, and enteroinvasive E. coli. Each of the virulent Shigellae and enteroinvasive E. coli have large plasmids comparable to that of YSH6000 (Figure 6A, lanes 1–6), which hybridized strongly with the virG probe (Figure 6B, lanes 1–6). The apparent hybridization to the chromosomal DNA of these strains can be attributed to contaminating sequences from the large plasmid, since chromosomal DNA from S. flexneri cured of the large plasmid, and from E. coli K12 (Figure 6, lanes 7 and 8), failed to hybridize. Thus, virG is considered to be a common region on the large plasmid found in all Shigellae and enteroinvasive E. coli.

Discussion

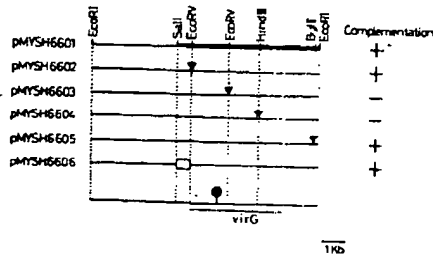Colonic epithelial invasion of Shigellae is the essential early step in pathogenesis of bacillary dysentery (LaBrec

Figure 5. Restriction Map of pMYSH6601 and virG Region Determined by Complementation Tests

▼, 4 bp or 8 bp insertion; ▢, deleted region. The thick line on the map of pMYSH6601 represents the 6.1 kb SalI–EcoRI fragment derived from SalI-G of pMYSH6000, and the thin line represents pMY6003 sequences. Each plasmid derivative was used to transform the Tn5 insertion mutant M94, and the results of complementation for virulence was judged by the mouse Sereny test. +, positive Sereny test, and –, negative Sereny test. ● represents the Tn5 insertion site of F24.
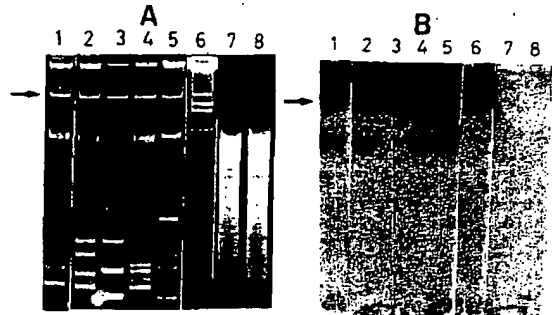

Figure 6. Southern Hybridization Test for the virG Sequence of Shigellae and Enteroinvasive E. coli

(A) Ethidium bromide-stained 0.7% agarose gel. (B) Autoradiogram of Southern blot hybridization on the gel A with the virG-derived fragment as probe. Lanes 1, S. flexneri 2a YSH6000; lanes 2, S. flexneri 98; lanes 3, S. dysenteriae 121; lanes 4, S. boydii 74; lanes 5, S. sonnei 75; lanes 6, enteroinvasive E. coli 282-83 FAV (L. L. Rabulsi); EcoRI-digested chromosomal DNA of YSH6000 cured of the large plasmid; lanes 8, EcoRI-digested chromosomal DNA of E. coli K12 MC1061. Arrows indicate the large virulence plasmids of 120 Md to 140 Md.

et al., 1964). This is assumed to occur through the induced phagocytosis following the bacterial attachment to the surface of epithelia. Subsequently, the bacteria multiply within the phagosome, destroy it, spread freely within the cytoplasm, and reinfect the adjacent epithelial cells. The twelve avirulent Tn5 insertion mutants described in this paper can invade and multiply but do not proceed further. Although multiplication occurs, the bacteria lack active movement, show a tendency to localize within the cytoplasm, are gradually converted to a spherical morphology, and are finally extinguished from the epithelia. These observations may suggest the following possibilities. First, the phagosome membrane may not be destroyed in these mutants. Second, these bacteria may have lost the ability to resist lysosomal bactericidal enzymes. Or they may have lost a factor required for normal growth within the epithelia. Mutant bacteria may have finally been extinguished through phagosome–lysosome fusion and bactericidal activity. This suggests that the function of the virG gene product is to resist somehow the bactericidal activity of lysosomal enzymes. This resistance may be exerted through lysis of the phagosome membrane. As in Rickettsiae (Ewing et al., 1978), shigellae may have to escape from the phagosome to avoid being killed by lysosomal enzymes.

The above observations were made in experiments conducted both in vitro and in vivo. This suggests that the Tn5 insertion mutants used in this study may invade colonic epithelia and multiply therein but are presumably unable to induce dysentery. In this sense they may be a plausible candidate for a live vaccine against bacillary dysentery.

A segment of about 45 kb from the large plasmid of S. flexneri 5 was cloned into a cosmid vector (Maurelli et al., 1985). This region is sufficient for invasion into HeLa cells, but not for plaque tests (Oaks et al., 1985) nor for positive Sereny tests (Maurelli et al., 1985; Sansonetti et al., 1986). These authors postulated that other determinant(s), besides those on the 45 kb fragment, are required to yield a positive Sereny test. The virG gene may be such a determinant. We have not yet done the plaque formation for virulence, but virG mutants are presumably

negative, because they are Sereny negative. By the use of the virG-defective avirulent mutant, we may be able to understand more clearly the mechanisms involved in producing a positive Sereny test, or reinfection after invasion into epithelial cells.

Experimental Procedures

Bacterial Strains and Plasmids

Fully virulent S. flexneri 2a strain YSH6000 was used as the wild type. Seventeen Tn5 insertion mutants into SalI-G of pMYSH6000 contained within YSH6000 were isolated independently among about 1000 insertions in the previous study (Sasakawa et al., submitted). S. flexneri 98, S. dysenteriae 121, S. boydii 74, S. sonnei 75, and enteroinvasive E. coli 282-83 FAV (0136) were used for DNA-DNA hybridization.

pMY6002 was generated by BglII linker insertion in pBR322 at position 3256 (Sutcliffe, 1979). The EcoRI-BglII fragment of pMY6002 was replaced by the EcoRI-BamHI fragment of R388, which contained a trimethoprim (Tp) resistance gene, and pMY6003 was constructed. The 6.1 kb EcoRI-SalI fragment within SalI-G of pMYSH6000 was inserted into the EcoRI-SalI site of pMY6003, and thus pMYSH6601 was constructed. pMYSH6602 and pMYSH6603 were constructed by inserting a SalI linker at the EcoRV site of pMYSH6601. pMYSH6606 was constructed by cleaving pMYSH6602 with SalI and self-ligating. pMYSH6604 and pMYSH6605 were constructed by digesting pMYSH6601 with HindIII and BglII, respectively, followed by treatment with the DNA polymerase I large fragment, thus making 4 bp insertions at each site of pMYSH6601 (Figure 5).

Media

Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, MI) with or without addition of agar was used for selection by Tp resistance. Other media were as described previously (Sasakawa et al., 1986).

Test for Invasion in Tissue-Cultured Monolayers

This was as described by Maurelli et al. (1984), except that the cells used were Rhesus monkey kidney epithelial (MK) cells designated as LLC-MK2 (Watanabe and Nakamura, 1985). In the standard invasion test, bacterial infection was made for 2 hr at 37°C followed by an additional incubation for 3 hr after removing extracellular bacteria by washing. In comparing Tn5 insertions for the time course of invasion, bacteria were infected for 40 min, and then the MK cells were kept for 0, 1, 3, 6, 9, 12, or 18 hr.

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In Vivo Infection with Shigellae

Bacterial cell suspensions were made as described previously (Sasa-kawa et al., 1986). Thirty microliter suspensions of either YSH6000 or YSH6202-M94 were dripped on one eye of each of two guinea pigs (clean Hartley strain). The eyes including palpebra and cornea were removed at 3 hr and 24 hr after infection, fixed in 10% buffered forma-lin, and embedded in paraffin. For the indirect immunofluorescence assay (Kurata et al., 1983), the sections were picked up on neoprene-coated slides and dried at 37°C for 2 to 3 days. Deparaffinized sections were treated with 0.25% trypsin at 37°C for 1 hr. S. flexneri 2a-specific rabbit antibody, a kind gift of Denka-Seikan Co., Tokyo, was applied at 1:20 dilution for the first step reaction, followed by application of fluo-rescein isothiocyanate (Miles YEDA Ltd., Rehovot, Israel)-conjugated anti-rabbit IgG (goat) at a dilution of 1:20 for the second step.

Other Experimental Procedures

Mouse Sereny test, isolation and characterization of plasmid DNA, and DNA–DNA hybridization were described previously (Sasakawa et al., 1983). Transformation was made by the method of Morrison (1977) for E. coli K-12 and by that of Curtiss (1981) for S. flexneri.

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V

V 1 Symbol for the element, vanadium. 2 Symbol for the unit, volt. 3 Symbol for valine. 4 volume.

V. vision.

V$_H$ The variable region of the immunoglobulin heavy chain.

v Symbol for the unit, volt. An incorrect symbol.

V Symbol for the quantities (1) volume, expressed in cubic meters; (2) potential energy, expressed in joules; (3) electrical potential or potential difference, expressed in volts; (4) luminous efficiency.

V$_D$ Symbol for physiologic dead space, expressed in cubic meters.

v 1 Symbol for the quantity, velocity, expressed in meters per second. 2 Symbol for the quantity, specific volume, expressed in cubic meters per kilogram.

VA visual acuity.

V·A Symbol for the unit, volt-ampere.

vaccigenous Vaccine-producing.

vaccin VACCINE.

vaccina VACCINIA.

vaccinable Susceptible to successful vaccination.

vaccinal 1 Pertaining to vaccine or vaccination. 2 Capable of conferring immunity or protection when administered by means of inoculation.

vaccinate To give vaccine by injection in order to produce active immunity to disease.

vaccination [L *vaccinus* (from *vacca* a cow + -*inus* -INE) relating to a cow + -ATION] The administration of vaccine by injection or orally in order to produce active immunity to disease.
autogenous vaccination A vaccination that utilizes a vaccine prepared from organisms obtained from the patient himself.
booster vaccination The administration of additional doses of vaccine, usually smaller than the initial dose, at specified intervals after the primary vaccination to maintain the individual's immunity to a disease.
jennerian vaccination Vaccination with virus from cowpox lesions to immunize persons against smallpox.
serum virus vaccination An obsolete technique in which a virulent virus is inoculated at the same time as an antiserum against the virus. It is now thought that complications may ensue, such as giant cell pneumonia secondary to measles vaccination by this technique.

vaccinationist 1 A person favoring the use of vaccination. 2 Favoring or promoting the use of vaccination.

vaccinator 1 A person who performs vaccinations. 2 Any instrument used in vaccination.

vaccine

vaccine [L *vaccinus* (from *vacca* a cow + -*inus* -INE) relating to a cow] An antigenic preparation used to produce active immunity to a disease. Vaccines may be living, attenuated strains of viruses or bacteria which give rise to clinically inapparent or trivial infections. Vaccines may also be killed or inactivated organisms or purified products derived from them. Formalin-inactivated toxins (toxoids) are used as vaccines against diphtheria and tetanus. Synthetic or genetically engineered antigens are currently being developed for use as vaccines. Also called *vaccinum, vaccin*. ● The use of cowpox (vaccinia) virus to give immunity to smallpox is the original vaccine from which current usage derives.

acne vaccine A vaccine prepared from acne bacilli and white or yellow staphylococci that was formerly used in the treatment of acne.

adjuvant vaccine A vaccine to which some form of immunopotentiator has been added, as a suspension on which antigens are adsorbed, a water-in-oil emulsion, or an emulsion containing killed mycobacteria (Freund's complete adjuvant) to increase antigenicity.

adsorbed diphtheria and tetanus toxoids and pertussis vaccine A sterile suspension of the precipitate derived from treating a blend of diphtheria toxoid and tetanus toxoid with alum, aluminum hydroxide, or aluminum phosphate and mixing it with pertussis vaccine, a killed suspension of *Bordetella pertussis*. The three components are present in proportions sufficient to confer immunity to each of the diseases. The mixture is used especially for prophylaxis of diphtheria, tetanus, and pertussis in infants and young children.

adsorbed pertussis vaccine A sterile bacterial fraction or suspension of *Bordetella pertussis* which has been precipitated or adsorbed by aluminum hydroxide or aluminum phosphate. It is given intramuscularly as an active immunizing agent against pertussis.

anthrax vaccine A vaccine prepared from nonencapsulated spores of *Bacillus anthracis* and used to immunize persons at high risk of exposure to anthrax, including veterinarians and some industrial workers, and animals at risk of developing anthrax.

antirabic vaccine RABIES VACCINE.

antirabies vaccine of Pasteur A vaccine prepared from a living attenuated rabies virus. According to the original method advocated by Pasteur, treatment of rabies consisted of daily subcutaneous injections of a suspension of rabbit spinal cord in physiologic serum over a course of 15–18 days, using progressively younger and therefore more virulent spinal cord. Preparations of desiccated spinal cords have been generally abandoned in favor of phenolized rabies vaccines such as Fermi's and Sem-

ple's vaccines, although variants of Pasteur's type of rabies vaccination are still used in some countries. Also called *Pasteur vaccine.*

antityphoid vaccine TYPHOID VACCINE.

attenuated vaccine A live bacterial or viral vaccine, carrying mutations that eliminate its pathogenicity but not its ability to elicit a protective immune response.

attenuated live measles virus vaccine A sterile preparation of an attenuated line of measles virus grown in a chick embryo cell culture. It causes a mild, noncommunicable infection in most individuals after a single dose, which produces an immunity to measles that lasts at least 10 years.

autogenous vaccine Bacteria (usually staphylococci) cultured from a patient, killed, and injected in the hope of eliciting strain-specific immunity.

BCG vaccine A preparation containing a dried, living culture of the Calmette-Guérin strain of *Mycobacterium tuberculosis* var. *bovis* (bacille Calmette-Guérin). It is used for immunization against tuberculosis and is usually administered intracutaneously. Also called *Calmette's vaccine, tuberculosis vaccine.*

brucellosis vaccine 1 A live vaccine used for the prevention of brucellosis in cattle, usually made from an attenuated strain (Strain 19) of the causative organism, *Brucella abortus.* 2 A vaccine made from killed *Brucella abortus* and Strain 19 of *B. abortus* for the prevention of *Brucella ovis* infection in sheep.

Calmette's vaccine BCG VACCINE.

canine distemper vaccine 1 A vaccine containing canine distemper virus which has been attenuated by growth in chick embryos or tissue culture cells. 2 A vaccine containing attenuated human measles virus which cross-reacts antigenically with canine distemper virus. Antibodies produced to the measles virus will neutralize the canine distemper virus. For defs. 1 and 2 also called *distemper vaccine.*

Castañeda's vaccine Typhus vaccine prepared according to Castañeda's rat-lung method.

Castellani's vaccine TYPHOID AND PARATYPHOID VACCINE.

Chantemesse vaccine A specially prepared typhoid bacterial protein which was instilled into the conjunctival sac to induce a pronounced allergic reaction in persons with typhoid fever. The vaccine and the test are now obsolete. See also OPHTHALMIC REACTION.

cholera vaccine A sterile suspension of specific strains of killed *Vibrio cholerae* used to produce immunity to cholera.

clostridial vaccine A vaccine used to prevent infection with a clostridial organism such as *Clostridium tetani, C. chauvoei,* and *C. septicum.* Formalin-killed and alum-precipatated cultures give long-lasting, effective immunity against the toxins responsible for the ill effects of the clostridia.

cowpox vaccine Smallpox vaccine obtained from cultures grown on the skin of cows. See also SMALLPOX VACCINE.

Cox vaccine TYPHUS VACCINE.

crystal violet vaccine A killed-virus vaccine formerly used in the prevention and control of hog cholera.

Dakar vaccine YELLOW FEVER VACCINE.

Danysz vaccine A preparation combining cultures of the normal human bowel flora. The vaccine was thought by some to exert an antianaphylactic effect in certain allergic states. It is now obsolete.

diphtheria vaccine DIPHTHERIA TOXOID.

diphtheria and tetanus vaccine An active immunizing agent against diphtheria and tetanus which consists of a mixture of diphtheria toxoid and tetanus toxoid.

diphtheria and tetanus toxoids and pertussis vaccine An active immunizing agent against diphtheria, tetanus, and pertussis which consists of a mixture of diphtheria toxoid and tetanus toxoid (prepared using formaldehyde as the toxoiding agent) and a sterile suspension of killed *Bordetella pertussis.*

Also called *DPT vaccine, triple vaccine.*

distemper vaccine CANINE DISTEMPER VACCINE.

DPT vaccine DIPHTHERIA AND TETANUS TOXOIDS AND PERTUSSIS VACCINE.

duck embryo vaccine A rabies vaccine prepared from rabies virus grown in duck embryonic tissue, inactivated with beta-propiolactone, and administered in a series of 14 daily intramuscular injections to persons exposed to rabies. This vaccine was widely used for 20 years but has been discontinued in the United States in favor of the more efficacious human diploid cell vaccine. Abbreviation: DEV

Durand's vaccine A typhoid-paratyphoid vaccine containing a microbial suspension with an admixture of 1:1000 diethyldithiocarbamate to potassium.

Durand and Giroud vaccine A vaccine against epidemic louse-borne typhus prepared from a culture of *Rickettsia prowazekii* grown on the lungs of white mice or rabbits and treated with formol. Vaccination consists of three subcutaneous injections of 1 ml each eight days apart, a booster injection being necessary one year after primary vaccination. The duration of immunity obtained is less than one year.

epidemic typhus vaccine TYPHUS VACCINE.

Felix vaccine A typhoid-paratyphoid vaccine containing bacteria inactivated by alcohol.

Fermi's vaccine A mixed attenuated-inactivated rabies vaccine containing a suspension of 5% sheep cerebral material inoculated with fixed rabies virus in an aqueous medium that contains at the outset 1% phenol, (reduced to 0.5% in the finished vaccine). Though habitually classed among inactivated vaccines, it includes, along with the phenol-inactivated virus, titrated quantities of live virus.

Flury vaccine An antirabies vaccine of chick embryo origin, of which there are two types: low egg passage (LEP) vaccine used in the vaccination of dogs, and high egg passage (HEP) vaccine used in the vaccination of other animals.

Flury HEP vaccine See under FLURY VACCINE.

Flury LEP vaccine See under FLURY VACCINE.

Haffkine's vaccine 1 A cholera vaccine prepared from a killed culture of *Vibrio cholerae.* It is administered in two injections 7–10 days apart. The first inoculation is weaker than the second. 2 A bubonic plague vaccine prepared from a killed culture of *Yersinia pestis.*

heterologous vaccine A vaccine based on one organism and used to protect another (for example, vaccinia virus and smallpox). An older term.

hookworm vaccine A vaccine formerly used to protect dogs against the hookworm, *Ancylostoma caninum.* It was made by preparation of x-irradiated larvae of the worm but is no longer available.

human diploid cell vaccine A rabies vaccine prepared from rabies virus grown in human diploid cell culture and inactivated. It is highly immunogenic and causes few reactions. This vaccine is now the only rabies vaccine licensed for use in man in the United States. Abbreviation: HDCV

humanized vaccine A smallpox vaccine prepared from material obtained from vaccinia vesicles in humans.

Idanov and Fadeewa vaccine A vaccine against measles prepared from attenuated virus grown in chick embryos. It is administered intradermally or subcutaneously.

influenza vaccine A killed or inactivated sterile suspension of influenza virus grown in embryonated chicken eggs. Specific antigenic types of influenza viruses A and B are used according to the type of influenza expected to be prevalent at a given time. The vaccine may be given intramuscularly or subcutaneously to enhance resistance to influenza virus infection. Attenuated living strains of influenza virus have also been used as vaccines. Also called *influenza virus vaccine.*

influenza virus vaccine INFLUENZA VACCINE.

jennerian vaccine SMALLPOX VACCINE.

Kelev's vaccine A rabies vaccine prepared from a live virus (Kelev strain) and analogous to the Flury HEP vaccine.

Kelser's vaccine An obsolete rabies vaccine prepared from rabbit brain inoculated with rabies virus.

Kolle's vaccine 1 A vaccine against plague prepared from sterile suspensions of *Yersinia pestis*. 2 A cholera vaccine prepared from killed cholera vibrios.

lapinized vaccine A vaccine which has lost its virulence for the original host by repeated passages through rabbits.

Lépine's vaccine A poliomyelitis vaccine analogous to the Salk vaccine and containing virus inactivated first by formol, then by β-propiolactone. It is administered subcutaneously in three injections, the second injection 6 weeks after the first and the third 2 to 3 months after the second. Vaccination is completed with booster injection given 1 year later.

live vaccine Any vaccine containing live microorganisms or viruses whose virulence has been attenuated.

live measles, mumps, and rubella virus vaccine A trivalent vaccine that contains an aqueous suspension of live, attenuated strains of measles virus, mumps virus, and rubella virus grown in chick embryo or duck embryo cells. The preparation is supplied as a lyophilized powder to be reconstituted immediately before use by adding a sterile diluent. It is used to obtain active immunization in children against all three viral diseases at the same time. Also called *MMR vaccine*.

live mumps vaccine A suspension of the Jeryl Lynn strain of mumps virus grown in chick embryo tissue culture. It is given subcutaneously to children older than 15 months and to adults. It brings about active immunity from a single injection and continuing protection against mumps for at least 10 years.

live oral poliovirus vaccine POLIOMYELITIS VACCINE.

lungworm vaccine A vaccine prepared from third stage larvae of *Dictyocaulus viviparus* that have been irradiated with x rays for the prevention of lungworm in cattle.

Lustig-Galeotti vaccine A plague vaccine consisting of *Yersinia pestis* and caustic soda neutralized by acetic acid.

Margulis and Sublaise vaccine A vaccine prepared in the USSR from a suspension of the cerebral tissue of mice infected with the virus of acute encephalomyelitis. The developers of this vaccine advocate its use in the treatment of sclerotic plaques. Its efficacy has not been demonstrated.

measles vaccine A live, attenuated measles virus vaccine obtained from cultures of the virus grown in chick embryos or on canine renal tissue. It is administered subcutaneously and confers active immunity against measles.

mixed vaccine A vaccine made from several different antigens of different pathogens.

MMR vaccine LIVE MEASLES, MUMPS, AND RUBELLA VIRUS VACCINE.

monovalent vaccine A vaccine made from a single dominant antigen of a given pathogen. Also called *univalent vaccine*.

multivalent vaccine A vaccine which contains antigens derived from several strains within a single species of pathogenic organisms. Also called *polyvalent vaccine*.

mumps vaccine A live, attenuated mumps virus vaccine obtained from cultures of the virus. It is administered subcutaneously and confers active immunity against mumps.

oral vaccine Any vaccine administered orally.

Otten's vaccine A vaccine of living, avirulent *Yersinia pestis* that is used in the active immunization against plague. This vaccine was employed in Java.

paratyphoid vaccine A vaccine prepared from killed cultures of one or more of the bacilli causing paratyphoid fever, *Salmonella paratyphi A, S. schottmuelleri*, and *S. hirschfeldii*. It is

given prophylactically to confer immunity against paratyphoid fever.

Pasteur vaccine ANTIRABIES VACCINE OF PASTEUR.

pertussis vaccine A sterile suspension or fraction of killed *Bordetella pertussis* administered to confer active immunity to pertussis. It may be combined with diphtheria and tetanus toxoids in a single trivalent injection. Also called *whooping cough vaccine, vaccinum pertussis*.

poliomyelitis vaccine 1 A sterile suspension of three types of inactivated polioviruses. The viruses are grown separately in monkey renal tissue cultures, and are then inactivated and combined. The vaccine is administered subcutaneously and confers active immunity against poliomyelitis. Also called *Salk vaccine*. 2 A suspension of one or a combination of three types of live, attenuated polioviruses propagated separately in monkey kidney tissue culture. The vaccine is administered orally and is an active immunizing agent against poliomyelitis. This vaccine appears to be more efficacious than the killed vaccine in preventing the spread of epidemic poliomyelitis. Also called *Sabin vaccine, live oral poliovirus vaccine*.

polyvalent vaccine MULTIVALENT VACCINE.

***Pseudomonas* vaccine** A vaccine made from the antigens of several strains of *Pseudomonas aeruginosa* and used prophylactically to immunize patients actively against future infection with *Pseudomonas* organisms. Its effectiveness in improving the chances for survival from massive burns is controversial.

rabies vaccine A sterile suspension of killed, fixed rabies virus obtained from duck embryo tissue previously infected with fixed rabies virus. The virus is inactivated by β-propiolactone. Rabies vaccine is also available prepared in human diploid cell tissue culture for those allergic to duck embryo vaccine or who do not develop adequate antibody titer to the latter. Also called *antirabic vaccine*.

rinderpest vaccine A vaccine prepared with a living virus which has been attenuated by either passage through goats or through tissue culture cell lines. It is used in cattle to prevent rinderpest (cattle plague).

Rocky Mountain spotted fever vaccine A sterile suspension of killed *Rickettsia rickettsii* which is prepared by growing the organism on chick embryos. The vaccine is administered subcutaneously as an active immunizing agent against Rocky Mountain spotted fever. However it has been shown to be of dubious value, conferring only limited protection against even small doses of infective rickettsiae. It is seldom used.

Sabin vaccine POLIOMYELITIS VACCINE.

Salk vaccine POLIOMYELITIS VACCINE.

Sauer's vaccine A pertussis vaccine derived from freshly isolated strains of *Bordetella pertussis*.

Semple's vaccine A modification of Pasteur's vaccine for rabies prepared from rabbit or sheep spinal cord infected with fixed rabies virus and inactivated with phenol. The vaccine was administered as a series of 14 daily intramuscular injections to give persons exposed to rabies a degree of immunity to the disease. It is now obsolete.

smallpox vaccine A suspension of living vaccinia virus obtained from cultures of a virus grown on the skin of vaccinated calves or sheep or on embryonated chick membranes. Available as a liquid or a freeze-dried preparation, the vaccine provides long-term active immunity against smallpox. In effective immunization, active viral infection is initiated in the skin by gently puncturing the skin with the side of a needle through a suspension of live virus. Also called *jennerian vaccine, vaccinum vacciniae, vaccinum variolae, variolovaccine*. ● This vaccine is no longer used, since smallpox has been eradicated throughout the world.

Spencer-Parker vaccine Rocky Mountain spotted fever vaccine prepared from ticks infected with *Rickettsia rickettsii*.

vaccine

vacuole

staphylococcus vaccine A vaccine prepared from cultures of one or more strains of *Staphylococcus*. It has been used in the treatment of some staphylococcal infections, especially recurrent furunculosis.

streptococcus vaccine A vaccine prepared from cultures of *Streptococcus* species.

Strong's vaccine A cholera vaccine prepared from nucleoproteins of *Vibrio cholerae*.

TAB vaccine TYPHOID AND PARATYPHOID VACCINE.

tetanus vaccine TETANUS TOXOID.

triple vaccine DIPHTHERIA AND TETANUS TOXOIDS AND PERTUSSIS VACCINE.

trivalent vaccine A vaccine with three strains or types of immunizing agent. For example, trivalent polio vaccine consists of polio immunotypes 1, 2, and 3.

tuberculosis vaccine BCG VACCINE.

tularemia vaccine A vaccine against tularemia prepared from cultures of *Francisella tularensis* inactivated by phenol, or after extraction by acetone, or from bacterial strains of little virulence. The protection conferred by the vaccine is not absolute.

typhoid vaccine A sterile suspension of heat- or chemical-killed *Salmonella typhi* in a diluent containing not less than one billion typhoid bacilli per milliliter. An active immunizing agent against typhoid fever, it is administered subcutaneously to persons at high risk for the disease (for example, persons in or traveling to endemic areas, health care personnel caring for typhoid fever patients). The degree of immunity conferred is not great. Oral vaccines of killed or attenuated typhoid bacilli have also been used. Also called *antityphoid vaccine, vaccinum typhosum, vaccinum antityphicum, typhobacterin.*

typhoid and paratyphoid vaccine A vaccine prepared from sterile suspensions of killed *Salmonella typhi, S. paratyphi A,* and *S. schottmuelleri* (formerly *S. paratyphi B*) and administered subcutaneously as an active immunizing agent to persons at high risk for typhoid and paratyphoid fevers. Also called *typhoparatyphoid vaccine, TAB vaccine, vaccinum typhosum et paratyphosum, Castellani's vaccine.*

typhoid-paratyphoid A and B, and cholera vaccine A mixture of sterile suspensions of killed *Salmonella typhi, S. paratyphi A, S. schottmuelleri* (formerly *S. paratyphi B*), and *Vibrio cholerae*. It is administered subcutaneously in an effort to confer immunity to typhoid and paratyphoid fevers and cholera in persons at high risk for these diseases.

typhoparatyphoid vaccine TYPHOID AND PARATYPHOID VACCINE.

typhus vaccine A sterile suspension of killed *Rickettsia prowazekii,* usually grown in embryonated chicken eggs. An active immunizing agent against epidemic louse-borne typhus, it is administered subcutaneously. Also called *Cox vaccine, epidemic typhus vaccine.*

univalent vaccine MONOVALENT VACCINE.

varicella vaccine Live attenuated varicella virus of the Oka strain, developed in Japan as a vaccine against varicella (chickenpox) and demonstrated to be effective in conferring protection as late as three days after exposure to infection and in children with malignancies. The vaccine has been shown to be 100% efficacious in preventing varicella in healthy children before exposure.

vole vaccine A vaccine prepared from cultures of *Mycobacterium muris,* the etiologic agent of tuberculosis in the vole. It has been used in tuberculosis prophylaxis in the same manner that BCG vaccine is employed.

Weigl vaccine A type of typhus vaccine prepared by infecting lice rectally with rickettsiae and emulsifying the louse intestines in a solution of phenol and sodium chloride.

whooping cough vaccine PERTUSSIS VACCINE.

yellow fever vaccine 1 A vaccine prepared from live, attenuated yellow fever virus grown in chick embryos and then freeze-dried. The reconstituted solution is administered subcutaneously to produce long-lasting immunity to yellow fever. 2 A vaccine prepared from mouse brain infected with the French neurotropic strain of yellow fever virus. It is administered topically by scarification. Also called *Dakar vaccine.*

Zinsser-Castañeda vaccine A typhus vaccine used in southern Mexico and derived from a combination of *Rickettsia prowazekii* and *R. mooseri* (murine rickettsiae).

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vaccinia [L. *vaccin(us)* (from *vacca* a cow + *-inus* -INE) relating to a cow + *-IA*] An acute infection caused by the vaccinia virus and characterized by a localized pustular eruption. The infection stimulates antibody production which confers immunity to smallpox. A live vaccinia virus preparation is used as an active immunizing agent against smallpox. When a viral infection of this nature occurs in cattle it is called cowpox. Also called *vaccina.*

chronic progressive vaccinia VACCINIA NECROSUM.

vaccinia gangrenosa VACCINIA NECROSUM.

generalized vaccinia A systemic infection which may occur about ten days after smallpox vaccination. The vaccinia virus appears in the bloodstream and a widespread vesicular rash erupts but leaves no scars. Also called *vaccinid, vacciniola.*

vaccinia necrosum A severe complication of smallpox vaccination characterized by progressive necrosis at the vaccination site and at metastatic sites due to unchecked viral growth. It usually occurs in immunologically deficient subjects. Also called *vaccinia gangrenosa, progressive vaccinia, chronic progressive vaccinia.*

progressive vaccinia VACCINIA NECROSUM.

vaccinial Pertaining to or of the nature of vaccinia.

vaccinid GENERALIZED VACCINIA.

vaccinifer VACCINOGEN.

vacciniform [*vaccini(a)* + -FORM] Vaccinialike; said especially of a rash.

vacciniola GENERALIZED VACCINIA.

vaccinization An outmoded practice of repeating vaccinations of an agent or virus until it is ineffective and immunity is complete.

vaccinogen The animal or other source from which vaccine is obtained. Also called *vaccinifer.*

vaccinogenous [*vaccin(e)* + o + -GENOUS] Producing vaccine or relating to its production.

vaccinoid Resembling vaccinia.

vaccinostyle A small pointed lancet used for vaccination.

vaccinotherapy The use of vaccine in the treatment of disease.

vaccinum [L. (substantive from *vaccinum,* neut. of *vaccinus* relating to a cow), vaccine] VACCINE.

vaccinum antityphicum TYPHOID VACCINE.

vaccinum pertussis PERTUSSIS VACCINE.

vaccinum typhosum TYPHOID VACCINE.

vaccinum typhosum et paratyphosum TYPHOID AND PARATYPHOID VACCINE.

vaccinum vacciniae SMALLPOX VACCINE.

vaccinum variolae SMALLPOX VACCINE.

vacuolar Related to or resembling a vacuole.

vacuolate Containing one or more vacuoles. Also *vacuolated.*

vacuolated VACUOLATE.

vacuolation [*vacuol(e)* + -ATION] The formation of development of vacuoles. Also called *vacuolization.*

vacuole [French (from L. *vacu(um)* vacuum + -*olum* -OLE), a small vacuum] 1 A minute clear region in the cytoplasm of a cell. 2 A membrane-limited chamber in the cytoplasm of a